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Heat Shock Proteins Volume 5

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Heat Shock Proteins and Whole Body Physiology

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HEAT SHOCK PROTEINS

Volume 5

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This book is dedicated to our children Dean, Diana, Dorte and Daffy (to B.K.P.) and Edwina, Vanessa and Alexzander Jr. (to A.A.A.)

PREFACE

The heat shock proteins (HSP) are a family of highly conserved proteins with critical roles in maintaining cellular homeostasis and in protecting the cell from chronically and acutely stressful conditions. HSP are molecular chaperones that participate in a variety of physiological processes and are widespread in organisms, tissues, and cells. It follows that chaperone failure will have an impact, possibly serious, on one or more cellular function, which may lead to disease. Activation of HSP results in stress tolerance and cytoprotection against otherwise lethal exposures to stress-induced molecular damage and the induction of HSP, therefore, may have broad therapeutic benefits in the treatment of various types of tissue trauma and disease. This book provides a comprehensive review on ccontemporary knowledge on the role of heat shock proteins in whole body physiology. Using an integrative approach to understanding heat shock protein physiology, the contributors provide a synopsis of novel mechanisms by which HSP are involved in the regulation of normal physiological and pathophysiological conditions.

Heat Shock Proteins and Whole Body Physiology reviews current progress on heat shock proteins in relation to diseases (Part I), psychological stress (Part II), exercise physiology and physiology of aging (Part III). Part I provides cutting edge knowledge regarding the regulatory role of HSP in the progression of a wide spectrum of diseases, ranging from diabetes, kidney diseases and cardiovascular diseases to infertility. Part II reviews our recent knowledge with regard to psychological stress, including learning, posttraumatic stress disorders, Alzheimer, social isolation and provides us with brand new information on the proteomics profile of chronically stressed individuals. Part III provides comprehensive reviews on the role of HSP in muscle. Increasing evidence suggests that intracellular expression of HSP has numerous protective effects for health and that increased muscular expression of HSP may represent one among several links between physical exercise and health. In contrast, HSP released during stress provoke pro-inflammatory responses and immune impairment. Finally, the "shock" of aging is presented. One of the key homeostatic responses involved in maintaining vitality and longevity is the induction of HSP. These chaperones play an important role in the deterrence of protein damage during aging.

Key basic and clinical research laboratories from major universities and hospitals around the world contribute chapters that review present research activity

and importantly project the field into the future. The book is a must read for researchers, postdoctoral fellows and graduate students in the fields of Endocrinology, Cardiology, Rheumatology, Physiology, Molecular Medicine, Aging, Pharmacology and Pathology.

Alexzander A. A. Asea and Bente K. Pedersen

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PART I

HEAT SHOCK PROTEINS AND DISEASE

CHAPTER 1

HSP AND DIABETES

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Abstract: As the prevalence of diabetes continues to rise, strategies that aim to prevent and treat the condition continue to gain importance. Obesity is thought to induce a state of low-grade inflammation, which ultimately disrupts insulin signalling and predisposes individuals to type II diabetes. In particular, TNFα, endoplasmic reticulum (ER) and oxidative stress all appear to be associated with obesity and stimulate inflammatory kinases such as c jun amino terminal kinase (JNK), inhibitor of NF-κβ kinase (IKK) and protein kinase C (PKC). These kinases in turn inhibit insulin signalling, predominantly through inhibitory phosphorylation of the insulin receptor substrate (IRS). The current chapter reviews the literature that describes this process and the potential that heat shock proteins have in preventing inflammatory disruption of insulin signalling. In particular, data are presented that demonstrate the role of Hsp72 in the prevention of insulin resistance in diet and genetic models of murine obesity. The role of HSP in the autoimmunity of type I diabetes is also discussed

Keywords: Obesity; inflammation; insulin resistance; hydroxylamine derivatives; autoimmunity

Abbreviations: β-HAD, β-hydroxyacyl-CoA-dehydrogenase; ATM, adipose tissue macrophages; BB, biobreeding; CS, citrate synthase; DAG, diacylglycerol; ER, endoplasmic reticulum; ERK1, extracellular signal-regulated kinases; FFA, free fatty acid; GSK-3β, glycogen synthase kinase 3β; HFD, high fat diet; HO-1, haem-oxygenase; HOMA-IR, homeostatic model assessment of insulin resistance; HT, heat therapy; HSE, heat shock element; HSF, heat shock factor; HSP, heat shock proteins; IKK, inhibitor of NF-κβ kinase; IMTG, intramuscular triglyceride; IPGTT, intraperotineal glucose tolerance tests; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-jun amino terminal kinase; MEF, mouse embryonic fibroblasts; NHANES, National Health and Nutrition Examination survey; NOD, non-obese diabetic mice; NSAID, non-steroidal anti-inflammatory drugs; OLETF, Otsuka Long-Evans Tokushima Fatty rats;

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PBA, 4-phenyl butyric acid; PERK, PKR-like kinase; PI, phosphatidylinositol; PIP_3, phosphatidilinositol 3, 4, 5 triphosphate; PKA, protein kinase A; PKC, protein kinase C; ROS, reactive oxygen species; TNFα, tumor necrosis factor α

INTRODUCTION

Diabetes is characterised by a chronic elevation of blood glucose (hyperglycaemia). The condition is broadly categorised into two types; type 1 is associated with insufficient insulin production, due to the destruction of pancreatic beta cells and type 2 encompasses a wide range of disorders that ultimately lead to hyperglycaemia. The persistent hyperglycaemia that results from these conditions can lead to further complications associated with the cardiovascular system, such as coronary artery disease, stroke and peripheral vascular disease. Although research into the pathogenesis of diabetes has progressed over the last 20 years, our understanding of this condition is still far from complete. Furthermore, the prevalence of diabetes is gradually increasing globally. Estimates report an incidence of 2.8% in the year 2000 and predict this value to grow to 4.4% by the year 2030 (Wild et al. 2004). Moreover, these projections assume other risk factors such as physical inactivity and obesity will remain constant, suggesting these figures could underestimate the global burden of diabetes. Given the apparent ubiquity of the highly conserved heat shock proteins (HSP), it is perhaps unsurprising that these chaperone proteins have been associated with a number of clinical conditions, including diabetes. HSP have been implicated in both the aetiology of immune mediated type I diabetes (Elias et al. 1990) and in the treatment of insulin resistance and obesity associated type 2 diabetes (Chung et al. 2008). The aim of this chapter is to review the current literature that implicates HSP as potential therapeutic targets for diabetes.

HSP derived their name following their apparent accidental discovery in the salivary glands of *Drosophila melanogaster* following transient heat stress (5°C above normal temperature) (Ritossa 1996). This heat shock response was characterised by the appearance of inducible proteins, which transiently gave rise to increased tolerance to high and otherwise lethal temperatures. Subsequently, the heat shock protein response has shown similar behaviour when faced with stressors such as oxidative stress, glucose deprivation and infection, which ultimately lead to the mis-folding of intracellular proteins. Therefore, the abundantly expressed HSP are primarily involved in cytoprotection, prevention of apoptosis and protein mis-folding and promoting signalling pathways during periods of cellular stress (Westerheide and Morimoto 2005). The heat shock protein superfamily have been traditionally organised by size and functional class. These include (alternative names in brackets (Kampinga et al. 2008)) Hsp100 (HSPH), Hsp70 (HSPA), Hsp60 (HSPD), Hsp40 (DNAJ), small HSP (HSPB) and haem oxygenase. The HSP60 and HSP70 families have received particular attention with regard to diabetes.

HSP MECHANISM OF INDUCTION

Hsp gene transcription is effectuated via a transcription factor called HSF1. In the unstressed state, HSP are bound to HSF1 maintaining it in an inactive, monomeric

state. Cellular stress causes dissociation of the HSP/HSF1 complex, which allows unbound HSF1 to translocate to the nucleus, convert to a trimeric complex and activate DNA binding activity at the heat shock element (HSE) promoter region of the hsp genes (Amin et al. 1988; Sarge et al. 1993). While this process is clearly key to hsp gene transcription, overexpression of HSF1 leads to heightened HSF1-HSE DNA binding in the absence of hsp70 gene expression (Zuo et al. 1995), suggesting further regulatory input is required. In particular, numerous protein kinases are thought to potentially phosphorylate HSF1 at various serine residues, through which the heat shock response can be influenced by cellular stress. For example, in response to thermal stress in human embryonic kidney cells, Polo-like kinase 1 phosphorylates HSF1 on Ser^{419} and is thought to regulate its nuclear translocation (Kim et al. 2005). Furthermore, non-steroidal anti-inflammatory drugs (NSAID) such as sodium salicylate cause the monomer to trimer transition (Jurivich et al. 1992), suggesting that kinases that are inhibited by NSAIDs such as RSK2, ERK, and IKKα might negatively regulate HSF1 activation (Wang et al. 2006). Indeed, Melling et al. (2006) were able to demonstrate a role for protein kinase A (PKA) in the suppression of ERK phosphorylation of HSF1 at Ser^{307} in rat cardiac tissue following the stress of exercise. Since ERK phosphorylation participates in the down-regulation of HSF1 transcriptional activity (He et al. 1998), this implicates PKA and ERK as key mediators of hsp gene expression, particularly following exercise. Moreover, both in vitro and in vivo experiments imply a facilitatory role of Glycogen synthase kinase 3β (GSK-3β) in the negative regulation of HSF1 (Chu et al. 1996; He et al. 1998).

HSP EXPRESSION IN INSULIN RESISTANCE AND DIABETES

Perhaps the first link between HSP and diabetes was introduced with the observation that in insulin resistant and diabetic patients, HSP expression was markedly altered. Muscle biopsies taken from type 2 diabetic patients showed significantly lower mRNA levels of the inducible isoform of HSP70, Hsp72, than those taken from non-diabetic controls (Kurucz et al. 2002). Furthermore, data collected in our laboratory supported this finding and demonstrated a marked relationship between both Hsp72 and Haem-oxygenase (HO-1) mRNA and insulin stimulated glucose uptake during a hyperinsulemic-euglycemic clamp in type 2 diabetic patients (Bruce et al. 2003). Interestingly, Kurucz et al. (2002) assessed the Hsp72 mRNA concentrations in monozygotic twins discordant for diabetes in an attempt to estimate the contribution of genetic or acquired factors in the relationship between Hsp72 and diabetes. Hsp72 mRNA was significantly lower in non-diabetic co-twins than aged matched healthy controls. However, these non-diabetic twin "halves" were also insulin resistant, making conclusions on the influence of genetic inheritance of low Hsp72 difficult. Furthermore, the authors were able to show a progressive decline in Hsp72 mRNA from insulin resistance, to impaired glucose tolerance, to diabetes, suggesting this defect in Hsp72 mRNA is an acquired abnormality rather than an inherited one. Assuming that Hsp72 mRNA levels reflect protein expression, these

data suggest that Hsp72 expression is low in type II diabetic patients. A key question that arises, is whether decreased Hsp72 expression is the cause or consequence of metabolic complications in diabetes?

EARLY SIGNS OF AN INVOLVEMENT OF HSP72 IN THE ETIOLOGY OF TYPE 2 DIABETES

Early reports of a decreased expression of Hsp72 in type 2 diabetes (Bruce et al. 2003; Kurucz et al. 2002) were supported by a preliminary study identifying Hsp72 as only 1 of 17 genes out of >5000, that were markedly lower in insulin resistance patients versus healthy controls (Patti et al. 2001, Abstract). Since no correlation is evident between skeletal muscle Hsp72 mRNA and fasting plasma glucose and insulin concentration (Kurucz et al. 2002), it is unlikely that altered Hsp72 expression is due to the chronic elevation of plasma glucose in diabetes. Instead, there are data that support the hypothesis that lowered Hsp72 expression is causally involved, at least in part, in the development of insulin resistance and type 2 diabetes. Early speculation considered that Hsp72 expression might be affecting insulin sensitivity through a direct interaction with GLUT4 (Kurucz et al. 2002). However, we have shown no reduction in GLUT4 gene expression in diabetic patients versus aged matched controls (Bruce et al. 2003). In the same study, we directly measured intramuscular triglyceride (IMTG) content in the muscle biopsy samples derived from type 2 diabetes patients and aged matched healthy controls. IMTG content was ~150% higher in the patient group. Allied to the finding of lowered Hsp72 mRNA expression in diabetes, these data provided a rationale for the examination of the role of HSP expression in the etiology of obesity induced insulin resistance.

OBESITY, INFLAMMATION AND INSULIN RESISTANCE

Before examining how HSP might alter obesity induced insulin resistance, it is first worth noting the magnitude of the problem of obesity and the potential mechanistic links between over-nourishment and diabetes. The most recently published surveys suggest that, in affluent and well-nourished societies, obesity levels have reached epidemic proportions. For example, figures from the National Health and Nutrition Examination survey (NHANES) in the USA suggested that the prevalence of obesity (BMI >30) in 2000 was 30.5%. This represents an increase of 7.6% from the figures collected during 1988–1994 (Flegal et al. 2002). Similarly, the prevalence of obesity is as high as 36.5% in some areas of Europe (Berghoefer et al. 2008). The causes of these trends are unclear, although it is likely that social, economic, and cultural changes lead to an imbalance in energy intake and expenditure. Given the fact that the prevalence of obesity and diabetes appear to be rising in tandem, it is unsurprising that obesity is identified as a significant risk factor for diabetes. The

exact mechanisms by which this connection is made have been the subject of a great deal of research in recent years.

Numerous lines of evidence suggest a link between obesity and inflammation. However, the traditional characteristics of inflammation do not apply to the obese condition. Inflammation is a classical response to injury, characterised by swelling, redness, pain and fever (tumor, rubor, dolor and calor). As such, the inflammatory processes are seen as acutely beneficial to the host. However, prolonged or chronic inflammation is associated with a cluster of metabolic diseases, including diabetes. Therefore, this aspect of inflammation is often referred to as "low grade" or meta-inflammation (Hotamisligil 2006). While the cascade of molecules involved in inflammation is complex, the pro-inflammatory cytokine, TNFα has conferred a prominent role in mediating downstream transduction cascades that affect insulin signalling. In landmark studies, Hotamisligil and colleagues were the first to demonstrate that TNFα was overexpressed in the adipose tissue of obese mice (Hotamisligil et al. 1993). Moreover, in loss-of-function experiments in obese mice, null mutations in the gene encoding TNFα and its' receptors, resulted in improved insulin sensitivity (Uysal et al. 1997). Experiments that involve the adipose tissue are of particular relevance, as it appears that this is the predominant site of obesity-associated meta-inflammation. Indeed, both functionally and biologically, adipocytes and immune system macrophages show a high degree of similarity (Wellen and Hotamisligil 2005). Perhaps most significantly, both these cell types co-localise in adipose tissue in obesity, and in rodents and humans, adipose tissue macrophages (ATM) accumulate with increasing body weight (Weisberg et al. 2003; Xu et al. 2003). Furthermore, after surgery-induced weight loss in morbidly obese patients, ATM infiltration decreased significantly (Cancello et al. 2005). Of added interest is the high correlation between ATM accumulation and measures of insulin resistance (Xu et al. 2003), adding further support to the contention that obesity induced insulin resistance is determined, at least in part, by inflammation originating from adipose tissue.

A multitude of metabolic stressors appear capable of inducing inflammatory signalling pathways. In addition to the established influence of extracellular TNFα (Hotamisligil et al. 1996), stressors originating from within the cell appear influential. For example, obesity places overload on the endoplasmic reticulum due to an accumulation of misfolded proteins, lipid oversupply and increased demand on the synthetic machinery (Ozcan et al. 2004). Indeed, in both high fat diet and genetic (*ob/ob*) models of murine obesity, indicators of ER stress such as PKR-like kinase (PERK) and eIF2α are significantly phosphorylated in liver extracts from obese animals versus lean controls (Ozcan et al. 2006). Elevated glucose metabolism can also cause an increase in reactive oxygen species (ROS) in the mitochondria. Interestingly, gene expression analysis has suggested a role for ROS in both TNFα and glucocorticoid models of insulin resistance (Houstis et al. 2006). Given that both ER and oxidative stress are known to induce inflammatory signalling cascades (Kamata et al. 2005; Ozcan et al. 2004), these stressors provide additional means by which obesity disrupts insulin signalling.

MECHANISMS OF INSULIN SIGNALLING

Insulin itself is the most potent physiological anabolic agent that promotes storage and synthesis of lipids, protein and of course, carbohydrates. While the basic regulated transport of glucose into the cell is mediated by the GLUT4 receptor, a complex cascade of signalling cascades exists that effects the actions of insulin (Chang et al. 2004). The foremost step of this process is the initial binding of insulin receptor (IR). The IR itself is a heterotetrameric transmembrane complex, the α unit of which binds insulin, and initiates phosphorylation of the IR β units on tyrosine residues. The resulting activation of the kinase associated with the β units sets forth a web of phosphatidylinositol (PI) 3 kinase dependent and independent downstream signalling cascades (Chang et al. 2004; Taniguchi et al. 2006). Briefly, IRS stimulation of PI-3 kinase produces phosphatidilinositol 3, 4, 5 triphosphate (PIP_3) which stimulates kinases such as PDK. PDK in turn activates a series of kinases that results in the activation of Akt and PKC and subsequent regulation of GLUT4 mediated glucose transport. It is thought that this process can also be stimulated via lipid raft microdomains through activation of Cbl and APS (Chang et al. 2004). So while the process of insulin signalling is clearly complex, the initial tyrosine phosphorylation of the insulin receptor substrate family (IRS 1–6) is highly significant (Chang et al. 2004). For example, IRS-1 and IRS-2 knock-out mice are markedly insulin resistant, underscoring the importance of the IRS family on insulin signalling (White 2002). Indeed, the tyrosine phosphorylation of IRS proteins appears defective in experimental and human models of insulin resistance (Wellen and Hotamisligil 2005).

INFLAMMATORY KINASES AND THE DISRUPTION OF INSULIN SIGNALLING

While it is becoming increasingly established that fatty acids, proinflammatory cytokines, ER stress and ROS can disrupt insulin signalling, in order to develop various therapeutic avenues, it is important to determine the specific mechanisms by which these stressors induce insulin resistance. In this regard, the inflammatory serine/threonine kinases c jun amino terminal kinase (JNK), inhibitor of NF-κβ kinase (IKK) and protein kinase C (PKC) have received attention. JNK belongs to the MAPK family of kinases and has emerged as a key regulator of metabolic alterations in insulin sensitivity. Indeed, three lines of evidence highlight this. (1) JNK activity appears elevated in both dietary and genetic models of obesity (Hirosumi et al. 2002; Prada et al. 2005). For example, rats fed a high fat "western" diet for 30 days showed significantly higher JNK activity in liver, muscle and hypothalamus tissue, versus controls (Prada et al. 2005). In addition, JNK phosphorylation is elevated in liver, muscle and adipose tissues taken from leptin deficient (*ob/ob*) mice, a commonly used genetic model of murine obesity (Hirosumi et al. 2002). (2) JNK is activated by FFA, TNFα, ER stress and ROS (Kamata et al. 2005; Nguyen et al. 2005; Ozcan et al. 2004; Yuasa et al. 1998), all of which are known to contribute to

insulin resistance. (3) Finally, JNK serine phosphorylates IRS-1 (ser^{307}) which disrupts IRS-1 and IR interaction (Aguirre et al. 2002; Hotamisligil et al. 1996). Indeed, the importance of JNK in the development of insulin resistance is emphasised by data from JNK-1 knock-out animal experiments, that show markedly decreased ser^{307} IRS-1 phosphorylation in obese JNK-$1^{-/-}$ versus obese wild-type mice. Significantly, obese JNK-$1^{-/-}$ mice also demonstrated markedly improved measures of whole body insulin sensitivity versus obese wild type controls (Hirosumi et al. 2002). Taken together, these findings suggest that JNK inhibition might provide a promising therapeutic avenue for diabetes.

Other inflammatory kinases that inhibit insulin signalling are IKK and PKC. Indeed, high doses of salicylates, which inhibit IKKβ and NFκβ, reverses insulin resistance in genetic and diet models of animal obesity (Yuan et al. 2001). Moreover, $IKK\beta^{+/-}$ transgenic mice showed consistently lower fasting glucose and insulin concentrations following a high fat diet when compared to wild-type counterparts. In keeping with the inhibitory effects of other inflammatory kinases such as JNK, overexpression of IKKβ in hepatocytes resulted in decreased insulin-stimulated IR and IRS-1 phosphorylation (Cai et al. 2005). Infusion of lipid emulsions in rats, a model for fatty acid induced insulin resistance, causes an increase in intracellular fatty acyl-CoA and diacylglycerol (DAG) (Yu et al. 2002). Raised concentrations of these lipid species is associated with an increased activation of PKCθ and heightened IRS-1 ser^{307} phosphorylation (Yu et al. 2002), suggesting a role for PKCθ in direct disruption of insulin signalling. Collectively therefore, these data outline a potential mechanism mediated predominantly by inflammatory kinases, which accounts for TNFα, fatty acid, ROS and ER stress induced insulin resistance. Therapies aimed to limit this meta-inflammation thus require further investigation.

HSP AND INFLAMMATION

A key feature of HSP is their ability to provide cytoprotection. Early experiments demonstrated that if cells were heat treated at 43°C, the number of cells surviving a subsequent insult of heat shock increased. Furthermore, this "acquired thermotolerance" was associated with the synthesis of HSP (Landry et al. 1982). Once it became understood that HSP could provide cytoprotection against other stressors, interest in their therapeutic value increased. For example, an upregulation of HSP has been associated with improved recovery from ischemia in cardiac tissue (Snoeckx et al. 2001) and protection against acute respiratory distress syndrome (Weiss et al. 2007). Of particular interest are data that imply a role for HSP in the inhibition of stress activated kinases and subsequent apoptotic pathways. For example, preheating of human leukemic cells led to reduced cell death following a subsequent heat shock, which was associated with an inhibition of JNK and p38 activation (Gabai et al. 1997). That this effect might be mediated by HSP was assessed using ectopic over-expression of Hsp72 in the human PEER cell line. Indeed, overexpression of Hsp72 suppressed the apoptotic and stress kinase activating effects of heat, osmotic

shock, H_2O_2 and UV irradiation (Gabai et al. 1997). Subsequent work using Hsp72 transfected mouse embryonic fibroblasts (MEF), suggested that Hsp72 suppresses the JNK signalling pathway through physical association and prevention of JNK phosphorylation by its upstream kinase SEK1 (Park et al. 2001). Similarly, HSP70 proteins have been implicated in the inhibition of IKKγ and subsequent formation of IKK complexes (Salminen et al. 2008). Given the importance of NFκβ in inflammation, the inhibition of its kinase IKK has particular therapeutic significance. For example, overexpression of Hsp70 protects rats against sepsis induced lung injury through an inhibition of the IKK complex (Weiss et al. 2007). Finally, Ozcan et al. (2006) have demonstrated that chemical chaperones such as 4-phenyl butyric acid (PBA) can limit ER stress and subsequent JNK mediated insulin resistance in genetic models of murine obesity and diabetes.

HSP72 AND THE PREVENTION OF INSULIN RESISTANCE

Meta-inflammation appears to disrupt insulin signalling and HSP appear to have the potential to inhibit inflammatory kinases. Therefore, there is a strong rationale to investigate the potential therapeutic role of HSP in insulin resistance. In particular, the inducible isoform of the HSP70 family, Hsp72 has been a specific focus. Interestingly, one preliminary report has suggested that heat therapy in general might have potential in the treatment of diabetes. Type 2 diabetic patients using a hot tub daily for 3 weeks have shown improvements in glycaemia by unknown mechanisms (Hooper 1999). In order to investigate the effects of heat therapy and Hsp72 induction on insulin resistance, we recently carried out a number of experiments (Chung et al. 2008).

Heat Therapy, JNK Phosphorylation and Insulin Sensitivity

Mice were subjected to either heat or sham therapy (control) whilst consuming a high fat diet (HFD). HT (heat therapy) involved raising the core temperature to 41°C for 15 min, once a week, for 16 weeks, which transiently increased Hsp72 expression in muscle, liver and adipose tissue. As expected, in response to the HFD, control mice developed hyperglycaemia, hyperinsulinemia and insulin resistance as indicated by the homeostatic model assessment of insulin resistance (HOMA-IR). Furthermore, intraperotineal glucose tolerance tests (IPGTT) revealed glucose intolerance in these mice. Conversely, mice exposed to HT were protected against insulin resistance, and this protection was associated with an attenuation of JNK phosphorylation in muscle.

Genetic Over-Expression of Hsp72 and HFD-Induced Insulin Resistance

While heat treatment showed improvements in insulin signalling in a high fat diet model of insulin resistance, the rather general nature of heat as a global stressor precludes firm conclusions on the involvement of HSP. Therefore, muscle specific

transgenic mice, overexpressing Hsp72 ($Hsp72^{+/+}$) were placed on HFD or standard chow diets and compared to wild type controls to determine the specific effects of Hsp72 expression on diet induced insulin resistance. In keeping with the heat therapy data, the development of hyperglycaemia, hyperinsulinemia, insulin resistance and glucose intolerance was prevented in $Hsp72^{+/+}$ mice as opposed to the WT controls. Given the role of inflammatory kinases in the disruption of insulin signalling, JNK and IKK phosphorylation was assessed in $Hsp72^{+/+}$ and WT mice. While neither the diet nor treatment altered IKKαβ serine phosphorylation, JNK (Thr^{183}/Tyr^{185}) phosphorylation was increased in WT mice following the HFD. Again, in keeping with the hypothesis, JNK phosphorylation was completely prevented in $Hsp72^{+/+}$ mice. Furthermore, when stimulated with insulin, akt phosphorylation was elevated in $Hsp72^{+/+}$ but not WT mice following the HFD. These data, therefore, indicated that overexpression of Hsp72 inhibited fatty acid disrupted insulin signalling, through the inhibition of the JNK pathway of inflammation.

Pharmacological Induction of Hsp72, JNK Phosphorylation and Insulin Resistance

Given the significance of the presented findings, it is important from a therapeutic point of view to determine ways in which Hsp72 can be induced. In this regard, hydroxylamine derivatives are thought to stimulate HSP expression by prolonging activation of HSF1 (Hargitai et al. 2003) and alteration of membrane lipid microdomains (Vigh et al. 2007). Therefore, the therapeutic value of the hydroxylamine derivative, BGP-15 was determined in a well-known model of obesity and diabetes, the *ob/ob* mice. Mice treated with BGP-15 by oral gavage demonstrated a significant increase in intramuscular Hsp72 compared with mice receiving control treatment (saline). In keeping with numerous previous findings, the increased Hsp72 expression was associated with decreased activation of JNK phosphorylation. Furthermore, BGP-15 treated mice presented with improved fasting glucose and insulin concentrations than control mice and a hyperinsulinemic euglycaemic clamp revealed markedly improved glucose disposal rate in the pharmacologically treated mice. These data demonstrate that BGP-15 is able to induce heightened expression of Hsp72 in genetic models of obesity and that this increased protection is associated with improved glycaemia and insulin signalling via suppression of JNK activation.

HSP72, Mitochondria and Insulin Resistance

Hsp72 is known to protect cardiac muscle against mitochondrial damage caused by ischemia reperfusion injury (Suzuki et al. 2002). In addition, heat therapy increases both mitochondrial enzyme activity and exercise endurance capacity in rats (Chen et al. 1999). Interestingly, a significant positive correlation between the mRNA expression of Hsp72 and mitochondrial enzyme activity has been observed in human

skeletal muscle (Bruce et al. 2003). It is important to note that in our recent study (Chung et al. 2008), we observed smaller fat pads in HSP72$^{+/+}$ mice compared with WT mice, even though the daily food intake was the same when comparing strains. This prompted us to examine the oxidative capacity in skeletal muscle of WT and HSP72$^{+/+}$ mice by measuring the maximal activities of two important mitochondrial enzymes, citrate synthase (CS) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD). Interestingly, the maximal activities of these enzymes was higher in HSP72$^{+/+}$ compared with WT mice. These data may suggest that Hsp72 increases the fatty acid oxidative capacity in skeletal muscle, which may account for the protection against increases in body weight and resultant insulin resistance.

To summarise, these data collectively imply Hsp72 as a potential target for the treatment of obesity induced insulin resistance. Regardless of the method used to overexpress Hsp72, heat treatment, genetic and pharmacological manipulation of this protein resulted in improved measures of insulin sensitivity in both high fat diet and genetic models of obesity. Furthermore, these improvements appeared to be tightly linked with a reduction in JNK phosphorylation. Therefore, it appears likely that Hsp72 may act to limit inflammatory kinase disruption of insulin signalling. Pharmacological induction of Hsp72 may therefore provide an attractive avenue for insulin resistance treatment in obese individuals (Fig. 1.).

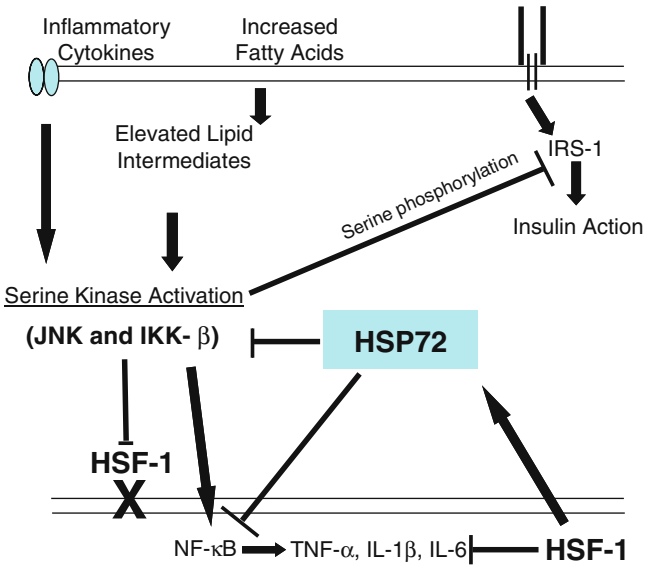

Figure 1. Schematic showing the interaction between inflammation, HSP regulation and insulin action. *Bold arrows* indicate pathway activation. *Blocked lines* indicate impaired signalling. Stress kinases are proposed to phosphorylate HSF-1 on specific serine residues preventing HSF-1 nuclear translocation (X) thus rendering HSF-1 transcriptionally silent. Increased HSF-1 activation and transcriptional competency leading to increased cellular Hsp72 levels strongly correlate with insulin sensitivity

HSP, JNK, & β CELL APOPTOSIS

Both type I and overt type 2 diabetes are essentially characterised by pancreatic β cell destruction or failure. For example, in non-obese diabetic mice (NOD), which quickly develop type I diabetes, apoptotic destruction of pancreatic cells is critical step in the development of the disease (Lee et al. 2004). To a lesser extent, reduction of β cell mass is also an issue in overt type II diabetes. Inadequate β cells may lead to the onset of diabetes in patients that have increased demand for insulin (e.g., obese and insulin resistant individuals) (Zhao et al. 2008). Interestingly, in Otsuka Long-Evans Tokushima Fatty (OLETF) rats that are often used as a model of obesity and type II diabetes, the rate of β cell apoptosis was significantly increased versus control rats (Zhao et al. 2008). While overall β cell mass increased, insulin signalling in these cells was impaired, suggesting that increased cell mass was a compensatory mechanism for overall β cell incompetence and peripheral insulin resistance. Interestingly, heat-induced apoptosis is correlated with activation of the SAPK/JNK pathway (Mosser et al. 1997), and cell death mediated by the sphingomyelin pathway can be prevented via inhibition of JNK (Verheij et al. 1996). Given the data that suggest HSP are able to disrupt the inflammatory kinases such as JNK (Gabai et al. 1997), treatments that improve HSP expression may help prevent β cell apoptosis in diabetes. Indeed, overexpression of Hsp72 in renal cells (Meldrum et al. 2003), T lymphocytes (Mosser et al. 1997) and sympathetic neurones (Bienemann et al. 2008) protects against apoptosis induced by ischemia, heat and growth factor withdrawal.

HEAT SHOCK PROTEINS AND THE "STRESS" OF DIABETES

That essential cells might be vulnerable in diabetes is another potential therapeutic avenue for HSP. For example, islet cells taken from the autoimmune diabetes-prone biobreeding (BB) rat show an increased vulnerability to oxidative stress, an alteration that can be limited by prior heat conditioning (Bellmann et al. 1997). Indeed, the heat shock protein response is a key aspect of cellular defence and therefore HSP expression might be important when attempting to deal with the "stress" of overt diabetes. For example, experimental induction of type I diabetes via streptozotocin treatment decreased Hsp60 expression in the myocardium of rats (Chen et al. 2005). Indeed, this decrease in HSP expression and cellular protection may well be an underlying mechanism behind diabetic cardiomyopathy (Shan et al. 2003). Reductions in Hsp72 have also been observed in liver and skeletal muscles from experimentally induced diabetic rats (Atalay et al. 2004). Interestingly, the decreases in HSP expression associated with diabetes can be offset by endurance exercise (Atalay et al. 2004) and antioxidant supplementation (Oksala et al. 2006). Given that similar decreases in HSP expression have been observed in human diabetes patients (Bruce et al. 2003), the maintenance of HSP expression in diabetes might be a further aspect of the acknowledged benefit of exercise on insulin resistance (Hawley 2004) and diabetes (Ostergard et al. 2007).

HSP AND AUTOIMMUNITY OF TYPE I DIABETES

The drop in insulin production associated with type I diabetes is essentially brought about by an autoimmune event that culminates in the destruction of the pancreatic islet β cells (Bach 1994). Interestingly, heat shock proteins have been mentioned as one of the potential antigens to which the autoimmunity develops. For example, in a mouse model of spontaneous autoimmune diabetes (NOD mice), the onset of β cell destruction was associated with anti-Hsp60 immunity (Elias et al. 1990) and T cell clones that recognise Hsp60 are able to transfer the development of insulinitis and hyperglycaemia in young prediabetic NOD mice (Elias et al. 1990). Identifying the target antigens that induce the autoimmune event is important in order to try combating the development of β cell destruction through immunotherapy. Indeed, vaccination with Hsp60 and an associated peptide, p277, has been successful in preventing spontaneous diabetes in NOD mice (Birk et al. 1996; Elias et al. 1991) and streptozotocin treated animals (Elias et al. 1994; Szebeni et al. 2008). It is thought that this immunotherapy might be utilised to alter the balance of immunity away from the Th1 pathogenic autoimmune response and toward a protective Th2 antibody response. Significantly, newly diagnosed child and adult patients with type I diabetes, also show a similar heightened autoimmunity to Hsp60, Hsp70 and p277 (Abulafia-Lapid et al. 1999, 2003). Although it should be acknowledged that the autoimmune event predisposing diabetes is asymptomatic, manipulation of HSP autoimmunity may provide future treatment avenues for diabetes. Indeed, Raz et al. have demonstrated improved preservation of β cell function in type I diabetes patients receiving regular doses of p277 derived from Hsp60 (Raz et al. 2001, 2007). Furthermore patients receiving p277 used 20% less insulin than non treated controls and the treatment caused no ill side effects. While it should be noted that the benefits of this treatment required constant doses, these data suggest that alteration of HSP auto-immunity has treatment potential in type 1 diabetes.

CONCLUSION

Diabetes and obesity are of a major public health concern. We have summarised here some of the currently known pathways through which obesity leads to insulin resistance and diabetes. While these mechanisms are clearly complex, current data suggests that HSP have the potential to alter obesity induced insulin resistance (Chung et al. 2008). HSP are also thought to be targets in autoimmune type I diabetes and an alteration of this autoimmune response can lead to improved clinical symptoms (Raz et al. 2007).

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CHAPTER 2

ROLE OF HEAT SHOCK PROTEINS IN OBESITY AND TYPE 2 DIABETES

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Abstract: Heat shock proteins (HSP) play an important role in human health and physiology and are known to function intracellularly as cytoprotection proteins by protecting cells against a wide variety of stressors, and extracellularly as chaperokines by stimulating the synthesis of pro-inflammatory cytokines, chemokines, and upregulates co-stimulatory molecule expression on antigen presenting cells, and enhancing natural killer (NK) cell-mediated migration and general anti-tumor responses. Obesity is known to be associated with raised serum inflammatory markers suggesting a state of heightened immune activation. The recent findings that antibody titers to several HSP are elevated in dyslipidaemic patients and individuals with established vascular disease, and that patients with Type 2 diabetes have reduced gene expression of Hsp72 which correlates with reduced insulin sensitivity point to an important role for HSP in obesity and Type 2 diabetes. This chapter briefly reviews recent advances in our understanding of the role of Hsp70 in obesity and Type 2 diabetes

Keywords: BMI; cancer; cardiovascular diseases; diabetes; obesity; weight

Abbreviations: BMI, body mass index; CDC, center for disease control; CRP, C-reactive protein; CVD, cardiovascular disease; FBG, fasting blood glucose; FFA, free fatty acids; GRP, glucose regulated proteins; HSF, heat shock factor; Hsp, heat shock proteins; *hsp*, heat shock protein gene; HSP, heat shock protein family; IGF-1, insulin growth factor-1; IL, interleukin; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinases; MCP-1, monocyte chemotactic protein-1; NK, natural killer; TNF-α, tumor necrosis factor-alpha; WHO, World Health Organization

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INTRODUCTION

Over the past decade, obesity has become a major public health problem in most industrialized nations (Pischon et al., 2007). Most health professionals now believe that obesity is a pandemic. Obesity is a complex, multi-factorial chronic disease that develops from an interaction of social, behavioral, cultural, physiological, metabolic and genetic factors (Nisoli and Carruba, 2002). Obesity is a condition in which the natural energy reserve is expanded far beyond usual levels to the point where it is believed to pose a health risk, and is currently measured using the body mass index (BMI). In the U.S., 26% of the population is obese, and an additional 36% are overweight. The obesity rates for the general U.S. population have increased dramatically over the past 40 years: children (6–11 years) increased from 4.2 to 18.8% (1963–2004); adolescents (12–19 years) increased from 4.6 to 17.4% (1966–2004); adults increased from 15 to 32.9% (1976–2004) (Ogden et al., 2002, 2006). It has been estimated that the costs associated with the management of obesity and obesity-related diseases account for about 5% of total healthcare expenditures in most industrialized nations (Thompson and Wolf, 2001).

There is a current trend that the obesity rates are higher for women than for men. Other risk factors for the development of obesity include poverty, lower education level and sedentary lifestyle (Ogden et al., 2006). More than one-third of U.S. adults were obese in 2005–2006, which includes men (33.3%) and women (35.3%). Healthy People 2010 define regular physical activity as exercise that promotes cardio respiratory fitness 3 or more days per week for 20 or more minutes per occasion. The Center for Disease Control (CDC) has identified several subpopulations at risk for lower rates of regular physical activity, including women (of all ages); people with low income; African-Americans and Hispanics; adults in the northeast and southeast; and people with disabilities.

During the past four decades, obesity has been identified as a major risk factor for the development of numerous health conditions including insulin resistance, Type 2 diabetes mellitus and cardiovascular disease (CVD) (Must et al., 1999; Flegal et al., 2002; Zimmet et al., 2005; Hotamisligil, 2006). The World Health Organization (WHO) currently defines diabetes as having fasting blood glucose (FBG) of ≥ 7.0 or ≥ 11.1 mmol/L 2 h after oral ingestion of a 75 g glucose load. To date, the level of blood hyperglycemia is the main criteria in defining Type 2 diabetes; the dysregulation of both insulin action and pancreatic beta cells is currently gaining more focus. This is important since a clear understanding of the disease and the mechanisms by which it exerts its negative effects is incompletely understood.

Heat shock proteins (HSP) are highly conserved proteins found in all cellular organisms. A wide variety of stressful stimuli have been shown to result in a marked increase in total HSP synthesis (Lindquist, 1986), known as the cellular stress response. The stress response is designed to enhance the ability of the cell to cope with increasing concentrations of unfolded or denatured proteins (Asea, 2005a). There are numerous HSP found in cellular organisms. However, the Hsp70 family constitutes the most conserved and best-studied class. The Hsp70 family consists

of the constitutively expressed Hsp70 (Hsc70; 73 kDa), the stress inducible Hsp70 (Hsp70; 72 kDa), the mitochondrial Hsp70 (Hsp75; 75 kDa), and the endoplasmic reticulum HSP70 (GRP78; 78 kDa) (Asea and DeMaio, 2007). The function of Hsp70 is exquisitely related to its structure. The Hsp70 family members all contain two major functional domains, including a N-terminal domain, also referred to as the ATPase domain which is composed of 45 kDa amino acids, and a C-terminal domain composed of a 15–18 kDa substrate-binding domain (SBD), and a 10 kDa carboxy-terminal domain of largely unknown function (Bukau and Horwich, 1998; Mayer and Bukau, 1998). A dichotomy now exists between the function of Hsp70 based on its relative location and the target cell it binds to and activates; intracellular versus extracellular (Asea, 2008a, b). Enhanced expression of intracellular Hsp70 increases the synthesis of anti-apoptotic proteins, which results in cytoprotection against a wide variety of stressors (Jaattela et al., 1998), represses gene expression (Tang et al., 2001), modulates cell cycle progression (Hut et al., 2005) and is anti-inflammatory (Housby et al., 1999). On the contrary, the increased release of Hsp70 to the extracellular milieu has recently been demonstrated to be immuno-stimulatory and results in the synthesis and release of pro-inflammatory cytokines (Asea et al., 2000, 2002; Asea, 2005b), augments chemokine synthesis (Lehner et al., 2000; Panjwani et al., 2002), upregulates co-stimulatory molecules (Asea et al., 2002; Bausero et al., 2005), induces NK cells migration and chemotaxis (Gastpar et al., 2005) and enhances anti-tumor surveillance (Srivastava et al., 1994; Srivastava, 2000, 2005).

The excessive body fat or adipose tissue found in most obese individuals was once thought to be a passive fuel storage component. However, it is now recognized as an endocrine organ with the ability to effectively communicate with the brain and peripheral tissues via secretion of bioactive mediators to regulate appetite and metabolism (Kershaw and Flier, 2004). Plasma free fatty acids (FFA) play important physiological roles in skeletal muscle, heart, liver, and pancreas. However, chronically elevated plasma FFA is linked with the onset of peripheral and hepatic insulin resistance (Park et al., 2008; Sheng and Yang, 2008). Although a great deal of work has been done on the role of Hsp70 in cancer and inflammation, only recently have researchers begun to elucidate the role of Hsp70 plays in insulin resistance and Type 2 diabetes, a major co-morbidity factor associated with obesity (Kurucz et al., 2002; Atalay et al., 2004). This chapter focuses on recent advances in our understanding of the role of Hsp70 in obesity and Type 2 diabetes.

BODY MASS INDEX (BMI)

BMI, also known as the Quetelet Index, is the mathematical formula used to calculate the body mass, given by the ratio of body weight (in kg) to body height (in m) squared (Expert-Panel, 1998), and is one tool used to define obesity. Currently an individual with a BMI $\geq$ 30.0 kg/m^2 is considered obese. The WHO guidelines issued in 1997 of a BMI of 18.5–25 kg/m^2 (acceptable range), up to 30 (overweight), and over 30 (obese) was based on data from western countries. BMI has been demonstrated to

correlate well with fat mass, morbidity and mortality, and is effective in reflecting obesity-related disease risk in a wide range of populations (Elia, 2001). The BMI was created in the nineteenth century by the Belgian statistician Adolphe Quetelet, and remained largely intact until June 1998 when the BMI was revised downward. This had the remarkable effect of changing some people's status from "ideal" weight to "overweight" in one day (Expert-Panel, 1998). BMI has been subject to fundamental criticism as it ignores fat distribution in the body and the fat/muscle proportion in total body weight in competitive athletes or body builders as they have a high BMI without necessarily having elevated health risks. The BMI weight categories generally do not apply to pregnant or nursing women, growing children or the elderly (Expert-Panel, 1998).

BMI AND OBESITY

Although BMI over 30 is considered obese in western countries, the body-frame rather than ethnicity is a likely reason for some of the differences between Asians and westerners in the relation between body fat and BMI (Saijo et al., 2004). It is concluded that the risk of obesity-related diseases among Asians rises from a BMI of 23, but there was no clear single cut-off point for all Asians for high risk (analogous to being obese). According to Mabel Yap, vice chairperson for the expert consultation and director of the research and information management division at the Health Promotion Board, Singapore, is that, except for individuals who are morbidly obese, the way of bringing down a BMI into the acceptable range is by losing fat, not by losing weight – i.e., through increased activity rather than weight-reducing drugs (Choo, 2002). In addition, BMI as an indication of obesity status is less accurate in older individuals (over 65 years of age) who tend to accumulate relatively more body fat and less lean body mass, i.e., muscle and bone because muscle tissue has a much higher metabolic rate than does fat tissue, older individuals generally develop lower metabolic rates and therefore, the gain in body weight obviously exaggerated (Rivlin, 2007). Also in women changes in body composition are very similar to those in men at menopause (Arguin et al., 2008). Exercise improves body composition among healthy elderly, both by reducing fat mass and by increasing bone and muscle mass, thereby helping to restore higher metabolic rates (Rivlin, 2007).

BMI AND HSP

It has been previously shown that antibody titers to several HSP are elevated in dyslipidaemic patients and subjects with established CVD (Ghayour-Mobarhan et al., 2007a, b). Obesity is known to be associated with raised serum inflammatory markers suggesting a state of heightened immune activation. In this study the association between indices of obesity and several HSP antibody titers in healthy subjects was performed. Subjects (n=170) were recruited from among employees at the University of Surrey and the Royal Surrey County Hospital, Guildford, UK. Of

these subjects, 35 were obese with a body mass index (BMI)≥30 kg/m^2 (19 male and 16 female subjects), 58 were overweight with 30>BMI≥25 kg/m^2 (36 male and 22 female subjects) and 77 were of a normal weight with BMI<25 kg/m^2 (31 male and 46 female subjects). Overall, obese subjects had significantly higher plasma anti-Hsp60 ($P<0.001$), anti-Hsp65 ($P<0.05$) and anti-Hsp70 ($P<0.05$) compared with overweight and normal weight subjects (Ghayour-Mobarhan et al., 2007a).

OBESITY AND INFLAMMATION

The association between obesity and high serum inflammatory markers suggests a state of heightened immune activation. Indeed, pro-inflammatory cytokines, hormones and adipokines play a role in the mediation of insulin resistance (Guzik et al., 2006; Hotamisligil, 2006). Adipocytokines are bioactive mediators released from the adipose tissue including adipocytes and other cells present within fat tissues. These include several novel and highly active molecules released abundantly by adipocytes like leptin, resistin, adiponectin or visfatin, as well as some more classical cytokines released possibly by inflammatory cells infiltrating fat, like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1) or chemokine (C-C motif) ligand 2 (CCL-2), IL-1 (Tilg and Moschen, 2006).

Among the pro-inflammatory cytokines, TNF-α has consistently been implicated in the development of insulin resistance. In rodent and human models of obesity and diabetes, expression of TNF-α is abnormally elevated in adipose tissue and skeletal muscle (Hotamisligil et al., 1993; Kern et al., 1995; Saghizadeh et al., 1996). More specifically, the expression of TNF-α in adipose tissue and skeletal muscle impairs tyrosine phosphorylation of the insulin receptor (Uysal et al., 1997). This impairment is the result of TNF-α mediated serine phosphorylation of Insulin receptor substrate-1 (IRS-1), particularly on ser307 residues. However, genetic deletion of TNF-α restores insulin receptor tyrosine phosphorylation, improving whole body insulin sensitivity and glucose tolerance (Uysal et al., 1997). Obesity is a known coronary risk factor associated with a heightened inflammatory state (Trayhurn and Wood, 2005).

The most abundantly expressed within the adipose tissue are leptin and adiponectin. Interestingly some reports indicate that adipocytes may also release the latter, more classical cytokines. Typical adipocytokines like leptin or adiponectin have been initially recognized through their role in the regulation of energy storage and homeostasis. For example, leptin, acting centrally within the central nervous system (CNS) (Kaminski et al., 2006) plays an important role as a negative regulator of appetite control (Konturek et al., 2004, 2005). Further research has shown that receptors for those proteins, are widely expressed, throughout the cardiovascular and immune system (Stallmeyer et al., 2001; Guzik et al., 2003; Kougias et al., 2005).

Obesity is accompanied by generalized inflammation, characterized by increased plasma C-reactive protein (CRP) levels as well as by dysregulated cytokine production by monocytes, lymphocytes and other immune cells (Ouchi et al., 2003).

In addition, inflammatory cytokines have been shown to activate mitogen activated protein kinases (MAPK), including c-jun N-terminal kinase (JNK) in the onset of insulin resistance (Hirosumi et al., 2002; Hotamisligil, 2006).

OBESITY AND CANCER

Cancer is a complex and multifactorial process as demonstrated by the expression and production of key endocrine and steroid hormones that intermesh with lifestyle factors (physical activity, body size, and diet) in combination to heighten cancer risk (Fair and Montgomery, 2009). Excess weight has been associated with increased mortality from all cancers combined and for cancers of several specific sites. Restriction of calories by 10–40% has been shown to decrease cell proliferation, increasing apoptosis through anti-angiogenic processes (Fair and Montgomery, 2009). However, the independent effect of energy intake on cancer risk has been difficult to estimate because body size and physical activity, which are strong determinants of total energy expenditure. The inhibitory effects of physical activity on the carcinogenic process are reduction in fat stores, activity related changes in sex-hormone levels, altered immune function, effects in insulin and insulin-like growth factors, reduced free radical generation, and direct effect on the tumor. In vitro studies have clearly established that both insulin and insulin growth factor-1 (IGF-1) act as growth factors that promote cell proliferation and inhibit apoptosis (Prisco et al., 1999; Khandwala et al., 2000; Le Roith, 2000; Lawlor and Alessi, 2001; Yee, 2001; Moschos and Mantzoros, 2002; Calle and Kaaks, 2004). Moreover, insulin also affects the synthesis and biological availability of the male and female sex steroids, including androgens, progesterone and estrogens, which have a central role in regulating cellular differentiation, proliferation, and apoptosis induction (Flototto et al., 2001). Hyperinsulinemia is also associated with alterations in molecular systems such as endogenous hormones and adipokines that regulate inflammatory responses (Calle and Kaaks, 2004). Obesity-related dysregulation of adipokines has the ability to contribute to tumorigenesis and tumor invasion via metastatic potential. Obesity is associated with an aggressive form of prostate cancer and with alterations in androgen and estrogen metabolism. It was hypothesized that changes in components of the sex steroid receptor axis may contribute to the clinical aggressiveness of prostate cancer in obese patients (Gross et al., 2009).

HSP AND TYPE 2 DIABETES

It has also been reported that patients with Type 2 diabetes have significantly higher anti-HSP titers compared with non-diabetic controls (Sims et al., 2002). For many years, a link between obesity and Type 2 diabetes has been assumed but not proven. For example, obese individuals have a greater than tenfold increased risk of developing Type 2 diabetes as compared to normal weight individuals (Must et al., 1999; Field et al., 2001). Recently, formal scientific study into the mechanism by

which obesity causes diabetes has begun to be performed. Stumvoll and colleagues demonstrated that Type 2 diabetes develops due to an interaction between insulin resistance and beta cell failure (Stumvoll et al., 2005). These authors implicated several other factors including glucose toxicity, lipotoxicity and obesity-derived cytokines in the development of Type 2 diabetes (Stumvoll et al., 2005). In a pilot study, a proteomic approach was used to uncover important biomarkers that might link obesity with diabetes. SERPINE 1, which is associated with cardiovascular pathway, was identified as a possible biomarker in the plasma of patients with either diabetes or obesity and with both obesity and diabetes (Kaur et al., 2009). Further, our comparative proteomic profiling of plasma samples from the individuals with obesity and diabetes revealed apolipoprotein B, angiotensinogen, complement components, ceruloplasmin, fibronectin, inter-alpha inhibitors and hemopexin as possible biomarkers, which might link obesity and diabetes (Kaur et al., 2009). Taken together, our data demonstrates a first step in understanding a link between diabetes and obesity and suggest that further studies using larger numbers of individuals will provide critical information which can then be used for early detection (Kaur et al., 2009).

Although a distinct connection between Hsp70 expression, inflammation and insulin resistance has not yet been established, several studies have reported associations between Hsp70 and states of insulin resistance and Type 2 diabetes (Kurucz et al., 2002; Bruce et al., 2003; Atalay et al., 2004). Interestingly, it has previously been reported that elevation of core body temperature in patients diagnosed with Type 2 diabetes improves insulin sensitivity and glucose homeostasis (Hooper, 1999). Core body temperature was raised (mean of 0.8°C above basal) in diabetic patients via hot-tub immersion, 30 minutes a day, 6 days a week for 3 weeks. Following hot-tub therapy, patients showed decreased body weight, FBG and glycosylated haemoglobin levels (Hooper, 1999). Although this study did not report any mechanism by which glucose homeostasis was improved, it provided initial evidence of an association between heat treatment and Type 2 diabetes. Taking this idea a step further, it has been suggested that Hsp70 proteins could be a key component in the protection against insulin resistance and Type 2 diabetes (Kurucz et al., 2002; Bruce et al., 2003; Atalay et al., 2004). From skeletal muscle biopsies of patients with Type 2 diabetes, mRNA concentrations of Hsp72 were shown to be significantly reduced (Kurucz et al., 2002; Bruce et al., 2003; Atalay et al., 2004), as much as 55% compared with healthy controls (Kurucz et al., 2002; Bruce et al., 2003; Atalay et al., 2004). In addition, patients displaying reduced Hsp72 mRNA concentrations also displayed reduced glucose disposal capability as measured by euglycemic-hyperinsulinemic clamp, indicating a correlation between Hsp72 and insulin resistance (Kurucz et al., 2002; Bruce et al., 2003; Atalay et al., 2004). In the context of mitochondrial oxidative capacity, diabetic patients also demonstrated an inverse relationship between Hsp72 expression and citrate synthase and β-histone acetyltransferase (β-HAT) activity (Kurucz et al., 2002; Bruce et al., 2003; Atalay et al., 2004). Taken together, Hsp70 proteins may have a potential role in the prevention of insulin resistance and Type 2 diabetes.

CONCLUSION

There has been a dramatic increase in the number of obese in the US. Interestingly, in a great number of cases individuals considered obese develop diabetes later in their lifetime. Our pilot study for the first time revealed SERPINE 1 is the possible biomarker of interest linked with not only obesity and diabetes but also cardiovascular pathway. Besides this, apolipoprotein B, angiotensinogen, complement components, ceruloplasmin, fibronectin, inter-alpha inhibitors and hemopexin, the possible biomarkers were also observed in patients with obesity and diabetes, which might link obesity and diabetes and suggested further needed to be elaborate. Heat shock proteins (HSP) play an important role in human health and physiology and are known to function intracellularly as cytoprotection proteins by protecting cells against a wide variety of stressors, and extracellularly as chaperokines by stimulating the synthesis of pro-inflammatory cytokines and chemokines. The recent findings demonstrate that antibody titers to several HSP are elevated in dyslipidaemic patients and individuals with established vascular disease. However, patients with Type 2 diabetes have reduced gene expression of Hsp72, which correlates with reduced insulin sensitivity point to an important role for HSP in obesity and Type 2 diabetes. Evidence of a role of HSP in the development of obesity and Type 2 diabetes is just underway.

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CHAPTER 3

MULTIFACETED ROLE OF HEAT STRESS PROTEINS IN THE KIDNEY

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Abstract: The kidney represents an ideal "laboratory" for assessing the role of physiologic stresses on stress proteins. This organ is equally well suited for assessing the protective effects of stress proteins against known renal insults. As a metabolically active organ that operates on the brink of "hypoxic disaster" and is capable of concentrating therapeutic agents to levels far higher than present in the circulation, the kidney is vulnerable to diverse stressors that include oxygen deprivation, ischemia, and nephrotoxin. Stress proteins exert potent stabilizing effects on epithelial cell architecture that represent reversible or "sublethal injury." Stress proteins also promote cell survival, partly by interrupting the apoptotic pathway that contributes to organ failure. HSPs target different checkpoints in the cell death pathway, often utilizing distinct functional domains within a single HSPs to exert multiple cytoprotective effects. In sharp contrast to their protective effects in the intracellular milieu, recent evidence shows that HSPs in the extracellular compartment are pro-inflammatory. Given the relative paucity of treatments available to prevent injury or promote renal recovery, manipulation of endogenous stress proteins represents a promising arena for defining new approaches to nephrologic problems that contribute to substantial human morbidity and mortality

Keywords: Apoptosis; BCL2; hypoxia; ischemia; nephrotoxins; osmotic stress

Abbreviations: A_1AR, A1 adenosine receptors; AIF, apoptosis inducing factor; AKI, acute kidney injury; AVD, apoptotic volume decrease; BUN, blood urea nitrogen; CAN, chronic allograft nephropathy; CKD, chronic kidney disease; EMT, epithelial to mesenchymal transformation; GGA, geranylgeranylacetone; GSK3β, glycogen synthase kinase 3-beta; HSE, heat shock element; HSF, heat shock factor; HSP, heat shock protein; LPS, lipopolysaccharide; NO, nitric oxide; ORE, osmotic response element; PI3 kinase,

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phosphatidyl inositide 3 kinase; ROS, reactive oxygen species; TonEBP, tonicity-responsive enhancer binding protein; UT-A, urea transporter-A

INTRODUCTION

The human kidney is an ideal test site to examine the physiologic and pathophysiologic roles of stress proteins. Even under normal physiologic circumstances, survival in the renal environment requires tolerance to pH extremes, marked changes in osmolality, ionic composition and severe hypoxia. The urine pH frequently falls to 5.0 (vs. 7.4 in the bloodstream), representing more than a 100-fold increase in hydrogenion concentration between the renal epithelial cell and the urinary lumen. Urine osmolality ranges from 50 to 1200 mOsm/L (vs. 300 mOsm//L in blood) and is associated with marked changes in the urine content of urea, sodium, chloride and potassium not encountered elsewhere in the body. In addition, the kidney has one of the highest oxygen extraction ratios of any organ, a reflection of the "work" required to reabsorb 98–99% of the 180 L/day of glomerular filtrate comprised of water and solutes. As a result, oxygen content in the renal medulla falls to as low as 10 mmHg (Brezis and Epstein, 1993)! This "normal" environment induces stress protein production in a manner that parallels the intensity of these physiologic stressors (Emami et al., 1991; Rauchman et al., 1997).

Investigation of the renal pattern of stress protein production has revealed unique insights into the protective role of stress proteins in osmotic stress (Burg et al., 2007), hypoxia (Turman et al., 1997) and pH changes (Morimoto, 1998). The kidney, particularly the renal cortex with the lowest intra-renal level of HSPs (Emami et al., 1991) is one of the most vulnerable organs to ischemia, toxins and inflammation that often result in varying degrees of organ failure, hematuria and/or proteinuria, collectively referred to as "acute kidney injury" (AKI). In many cases, these pathophysiologic challenges induce additional stress protein expression, suggesting that the insulted kidney adapts to non-lethal stress by "anticipating" the next insult. The capacity of the kidney to rapidly up-regulate stress proteins after one insult and at the same time acquire resistance to injury from a second, unrelated insult characterizes the concept of "cellular cross resistance" and is likely mediated by these cytoprotective proteins. Finally, the kidney is unusual in its ability to survive an insult so extreme that renal function is completely lost, temporarily requiring renal replacement therapy to permit survival of the patient until renal function recovers. In most cases, recovery of organ function is associated with marked up-regulation of stress proteins, suggesting that these proteins participate in this highly regulated process. The proliferation of renal transplantation as a therapy for chronic renal failure has created another milieu in which renal stress and the role of stress proteins is being intensively investigated.

In a "natural experiment," investigators detected a significant difference in the sensitivity of tubules isolated from mature *vs.* immature rodent kidneys to stress. Specifically, the immature nephron exhibited greater tolerance to anoxic, heat, and oxygen stress that positively correlated with the increased capacity of immature

tubules to express Hsp72, the major inducible member of the HSP70 family (Gaudio et al., 1998). Similarly, the Brown-Norway rat, a species with a high level of Hsp70 in the kidney, is relatively resistant to transient ischemia (Basile et al., 2004). Although a positive correlation between Hsp72 content and cytoprotection has been shown, some studies have not established a causal relationship between these two parameters. However, many of the insults that induce Hsp70 expression are fortuitously ameliorated by prior Hsp70 induction, suggesting that indeed, Hsp70 and cell injury are intimately linked.

Recently, the exclusive identity of HSPs as versatile cytoprotectants have been challenged. While most investigators strongly support the notion that intracellular HSPs (particularly Hsp70 and Hsp27) promote cell survival, the release of these same HSPs into the extracellular environment may have the opposite effect. In fact, mounting evidence suggests that cytoprotective HSPs such as Hsp70 act as "chaperokines" once outside the cell, serving as key regulatory proteins in the inflammatory pathways that involve toll-like receptors (Asea, 2008). Surprisingly, some cells appear to have specific HSPs transporters, suggesting that frank cell lysis is not required to promote their escape (Bausero et al., 2005). Taken together, these reports show that HSPs serve dual roles that depend in large part on their location. These exciting findings confirm that the role of select HSPs in renal inflammation deserves increased attention.

This chapter reviews the principal renal disease states in which stress proteins have been implicated (Borkan and Gullans, 2002; Kelly, 2005; van de Water et al., 2006) and highlights areas for future investigation. In addition, studies that identify the role of stress proteins in renal cell injury and cell death pathways are discussed. The role of two well-characterized cytoprotective proteins, Hsp27 and Hsp70 is emphasized.

RENAL EPITHELIAL CELL INJURY, DEATH AND ORGAN DYSFUNCTION

The response of the kidney to stress is remarkable since sublethal and lethal cell injury both contribute to organ dysfunction. Historically, the single cell layer lining the renal tubules (the proximal tubule epithelial cell) has been identified as highly vulnerable to common insults such as ischemia or toxin exposure. Although renal epithelial cell necrosis has long been emphasized as the pathognomonic lesion in these insults, the amount of necrosis detected in human renal tissue fails to predict renal function or the potential for recovery in native kidneys or renal allografts (Rosen and Heyman, 2001). The lack of correlation between classic cell necrosis and organ function implicates sublethal cell dysfunction and/or alternative forms of cell death as contributors to organ failure. Indeed, renal epithelial cells are markedly sensitive to (potentially reversible) changes in the integrity of the actin cytoskeleton and cell contact sites caused by stress. The actin cytoskeleton and cell contact sites in turn regulate polarity that is required for the vectoral

solute and water transport. In addition, renal epithelial cells require an extensive tubulin-based microfilament structure that supports the structurally complex apical brush border that sustains solute reabsorption. In addition to sublethal injury, investigators have increasingly recognized that renal epithelial cell death follows more than a single pathway that includes both apoptosis and autophagy. The delayed recognition that non-necrotic forms of renal cell death contribute to organ dysfunction likely relates to their stochastic nature, their transient existence and the relatively insensitive detection techniques (Bonegio and Lieberthal, 2002). It now seems clear that organ dysfunction is also caused by cell injury to non-tubular target sites including the renal endothelium and vasculature (Sutton et al., 2002) and is promoted by inflammation (Daemen et al., 1999). Although many laboratories, including our own, have detailed the role of stress proteins in apoptosis, their role in cell autophagy (Ryhanen et al., 2008) and endovascular injury (Ortiz et al., 2004) remains largely unexplored. In addition, the relative contribution of lethal and sublethal renal cell injury to organ function remains to be clarified.

HSP AND RENAL CELL NECROSIS

In humans, renal cell necrosis presents only under extreme pathological conditions and is characterized by rapid decline in the glomerular filtration rate that leads to retention of waste products such as blood urea nitrogen (BUN) and creatinine and, often, decreased urine output. Unfortunately, morbidity and mortality in these situations remains dismally high despite significant scientific and technological advances, suggesting that renal injury also initiates untoward systemic complications (Kelly, 2006). Renal cortical necrosis usually results from prolonged renal ischemia, venom toxins, massive hemorrhage, high doses of nephrotoxins, or overwhelming sepsis and is typically extensive, although focal and localized forms do occur (Prakash et al., 2007). Acute ischemic renal failure often includes only "patchy" necrosis, most pronounced in the outer medulla in the S_3 segment of the proximal tubule, and results in exposure of denuded regions of basement membrane, retraction and/or shedding of the proximal tubule cell brush border and intraluminal cast formation. Together, these pathologic lesions permit backleak of glomerular filtrate, decrease the number of epithelial cells contributing to vectoral solute transport (i.e., to renal function) and precipitate intratubular obstruction, causing single nephron drop out. On a cellular level, classic necrosis is a passive, non-energy-dependent process characterized by rapid cell swelling, mitochondrial changes, and cell rupture that permits the release of cytoplasmic contents into the interstitial surroundings that in turn causes inflammation and additional tissue damage. Although necrosis has long been viewed as a non-regulated process, unresponsive to cytoprotective proteins, in non-renal cells this long-held concept has been challenged (Yaglom et al., 2003). In renal cells, the events that regulate necrosis other than severe or protracted adenine nucleotide depletion per se (Eguchi et al., 1997; Lieberthal et al., 1998) are uncertain and require additional investigation.

Renal ischemia-reperfusion injury triggers up-regulation of several intracellular signal proteins including the "classic" stress proteins. Microarray analysis of pre- and post-ischemic rat kidneys revealed 21 genes with a more than a threefold increase in expression after stress. Up-regulated genes included Hsp70 (43-fold) and Hsp27 (12-fold). Other proteins with potential cytoprotective roles are also induced, including heme oxygenase-1 (tenfold), kidney injury molecule-1 (eightfold), and several subtypes of S100 calcium-binding proteins (3.1- to 7.5-fold), suggesting that these molecules mediate the renal cell response to ischemia (Zhang et al., 2008). Interestingly, graded degrees of ATP depletion stimulate Hsp70 in the intact rodent kidney (Van Why et al., 1994), showing that the stress response is linked to adenine nucleotide content.

Heavy metals such as mercury or cadmium chloride are potent nephrotoxins that cause acute tubular necrosis and induce the synthesis of several cytoprotective molecular chaperones (Goering et al., 2000). Although heavy metals cause protein denaturation (DalleDonne et al., 1997), a potent stimulus for chaperone induction (Mifflin and Cohen, 1994), other signal events likely contribute to their induction. In fact, several investigators have shown that non-chaperone functions (i.e., independent of protein binding and refolding domains) participate in cytoprotection (Gabai et al., 2002; Ruchalski et al., 2006; Yaglom et al., 1999). Thus, it is likely that the pathways regulated by cytoprotective HSPs result not only from the burden of non-native proteins induced by diverse stressors, but by other intracellular events as well. In the normal kidney, Hsp65 is found in the cytoplasm of podocytes and proximal convoluted tubules, whereas Hsp70 is located in nuclei and cytoplasm of podocytes, cortical convoluted and collecting tubules (Hernadez-Pando et al., 1995). In a model of acute tubular necrosis caused by inorganic mercury, the distribution of both HSPs change as a function of time. In parallel with the appearance of tubular necrosis, Hsp60 immunoreactivity increased in all tubular structures of the cortex, most strongly in the pars recta of the proximal tubule. Damaged cells showed a shift of Hsp65 to the mitochondria and nucleoli, while Hsp70 was up-regulated in the cytoplasm, mitochondria, lysosomes, cytoskeleton, chromatin, and nucleoli. Interestingly, HSPs translocation coincided with their excretion into the urine (Hernadez-Pando et al., 1995), suggesting that detection of select HSPs in the urine may have diagnostic utility.

A1 adenosine receptors (A_1AR) prevented renal tubular necrosis-induced cell death both in vivo and in vitro (Lee et al., 2007). Surprisingly, expression of A_1AR in porcine renal tubule cells resulted in a significantly higher baseline expression of both total and phosphorylated Hsp27. The latter is likely due to A_1 receptor enhancement of p38 and AP2 mitogen-activated protein kinase activities. Compared to control, A_1AR expressing cells were more resistant to peroxide-induced necrosis as well as tumor necrosis factor-alpha and cycloheximide-induced apoptosis. A selective HSPs inhibitor increased cell death associated with adenosine receptor expression. In contrast, A_1AR receptor knockout in mice resulted in a decreased baseline level of Hsp27 and blunted adenosine-mediated HSPs phosphorylation.

These studies show that A_1AR activation protects renal proximal tubules partly by modulating Hsp27 signaling pathways (Lee et al., 2007).

In cultured human kidney cells, exposure to gentamicin, a nephrotoxic antibiotic, increased Hsp70 transcription and translation (Komatsuda et al., 1993), suggesting that Hsp70 either responds to gentamicin-induced cell stress or modifies toxicity caused by this agent. In proximal tubule cells, gentamicin-induced acute tubular injury causes Hsp73 (the constitutively expressed member of the HSP70 family) to translocate from the nucleus and accumulate in lysosomes. At the same time, Hsp73 shifts from a detergent-soluble into a detergent-insoluble protein fraction as might be predicted if cell proteins acquired a non-native conformation. These observations suggest that gentamicin-induced acute tubular injury induces protein denaturation, requiring Hsp73 to facilitate their lysosomal degradation (Komatsuda et al., 1993), an HSP70 function previously reported by others (Agarraberes and Dice, 2001).

OXIDATIVE STRESS/REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are small inorganic and organic ions that include oxygen ions, free radicals, and peroxides. These are highly reactive molecules due to the presence of unpaired valence shell electrons that react with double bonds in proteins, lipids, carbohydrates, and nucleotides. ROS form as by-products of normal metabolism and have important roles in cell signaling. However, oxidative stress markedly increases ROS to a level that exceeds local antioxidant capacity, causing target macromolecule oxidation and subsequent cell damage.

In the kidney, ROS are produced by several sources, including NAD(P)H oxidase, auto-oxidation of glucose, mitochondrial respiratory chain deficiencies, advanced glycation, peroxidases, xanthine oxidase and nitric oxide synthase. Oxidative stress results in increased production of free radicals such as superoxide ($\cdot O_2^-$) and hydroxyl radicals ($\cdot OH$), and non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl). Reactive nitrogen species produced by similar sources are capable of damaging renal cells and exist as either radicals (nitric oxide ($\cdot NO$) and nitrogen dioxide ($\cdot NO_2^-$)) or as non-radicals (peroxynitrite ($ONOO^-$) and nitrous oxide (HNO_2)). Cells are normally able to defend themselves against ROS damage with protective enzymes such as superoxide dismutases, catalases, glutathione peroxidases and peroxiredoxins. Small molecules such as glutathione, vitamin C, vitamin E and uric acid also play important roles as cellular antioxidants (Forbes et al., 2008).

Mounting evidence suggests that oxidative stress plays an important role in the development of renal fibrosis and progression of chronic kidney disease (CKD) characterized by the loss of organ function. Renin-angiotensin antagonism, calcium channel antagonism, n-3 polyunsaturated fatty acid, and other antihypertensive and anti-proteinuric therapies are commonly recommended for treatment of CKD.

Interestingly, some of these medications reduce oxidative stress in the kidney (Agarwal, 2003) and slow the progression of CKD. A link between the therapeutic effect of these agents and the renal stress response has not been completely examined.

Unilateral ureteral obstruction results in increased oxidative stress in the kidney leading to tubulointerstitial fibrogenesis and progressive loss of renal function. In a rat model of obstructive uropathy, losartan, an antihypertensive agent, decreased oxidative stress and fibrosis, independent of its effect on blood pressure. Importantly, protection was linked to increased Hsp70 expression (Manucha, W. 2005). Similar observations were made in human studies where oxidative damage to urinary proteins and lipids were reduced with additional angiotensin II receptor blockade independent of reductions in proteinuria or blood pressure (Agarwal, 2003). Of potential importance, we have recently linked renal epithelial to mesenchymal transformation (EMT) and renal fibrosis with Hsp70 expression. In this study, Hsp70 expression in cultured renal cells decreased TNF-beta1 induced EMT and reduced fibrosis in response to chronic ureteral obstruction in the intact rat kidney (Mao et al., 2008).

Exposure of kidney cells to $HgCl_2$ and $CdCl_2$ also generates a substantial oxidant stress from endogenous hydrogen peroxide that originates partly from damaged mitochondria (Nath et al., 1996). In addition to mitochondrial injury, the lysosomal proton gradient required for normal protein degradation is disrupted by heavy metals. Likely as a response to mitochondrial injury and the protein-damaging effects of hydrogen peroxide, molecular chaperones are induced. Acute heavy metal exposure increases the de novo synthesis of Hsp70 and Hsp90 in vitro and in vivo, suggesting that these HSPs may protect against the increased burden of denatured proteins caused by oxidative stress itself (Goering et al., 1992; Kim et al., 2000; Nath et al., 1996).

HSP AND SUBLETHAL RENAL INJURY

Disruption of epithelial cell architecture precedes and may in fact contribute to cell death (Borkan et al., 1997; Molitoris, 1991; Molitoris et al., 1991, 1997; Molitoris and Marrs, 1999). Both cell de-energization in vitro (induced by exposure to metabolic inhibitors that deplete ATP) and renal ischemia in vivo induce early, dramatic changes in the integrity of the actin cytoskeleton, disruption of cell-cell contact sites and loss of cell adhesion to the substratum. Consequently, cell polarity and vectoral solute transport are compromised. Tight junction function is altered leading to increased paracellular solute leak. Disruption of cell junctions permits viable renal epithelial cells to be shed into the urine of animals and humans (Racusen, 1998), demonstrating that sublethal cell injury contributes to organ dysfunction after ischemia. In addition, these "sublethal" changes may promote epithelial cell death. In non-renal cells, disruption of cell architecture (specifically to microtubules) liberates Bim, a pro-apoptotic protein that promotes cell death (Puthalakath et al., 1999). In

addition, collapse of the actin cytoskeleton results in macromolecular complexes of denatured actin filaments that bind Hsp70 (Kabakov and Gabai, 1993; Margulis and Welsh, 1991) or Hsp27 (Shelden et al., 2002; Smoyer and Ransom, 2002), potentially depleting these cytoprotectants from other key protein targets. Both Hsp70 and actin belong to a superfamily known as actin related proteins ("ARP"; Bork et al., 1992). In theory, this would permit Hsp70 to bind to monomeric actin, thereby limiting the formation of actin aggregates. Hsp27 also binds actin, acts as an actin capping protein (Shelden et al., 2002; Smoyer and Ransom, 2002) and regulate actin cytoskeletal changes in renal glomerular epithelial cells subjected to stress in which F-actin dysruption is of primary importance in sublethal cell injury (Smoyer and Ransom, 2002).

Studies examining Hsp27 distribution in sections of normal rat kidney show co-localization of Hsp27 with actin filament arrays, most notably at or near the apical microvillar border of proximal tubule epithelial cells (Aufricht et al., 1998a). Ischemia causes Hsp27 to closely associate with the actin cytoskeleton in proximal convoluted tubule cells (Schober et al., 1998). Similar observations were made in porcine kidney renal epithelial cells subjected to ATP depletion (Van Why et al., 2003). In cultured canine renal epithelial cells (MDCK) subjected to either heat-stress or ATP-depletion, GFP-labeled Hsp27 associates with the cytoskeleton. In these injured cells, Hsp27 localizes to the basolateral but not the apical cell border, suggesting that it may regulate epithelial cell-cell and cell-substrate attachments. Given the close relationship between the cytoskeleton and cell contact sites, it is not possible to determine with certainty whether these two effects are independent or linked to one another. However, the fact that Hsp70 reduces stress-mediated denaturation of paxillin, a key adaptor protein selected as a surrogate marker for focal adhesion complex integrity, suggests that Hsp70 exerts protective effects on the cytoskeleton and cell-contact sites and preserves renal cell attachment during stress (Mao et al., 2004). Perhaps as a result of their stabilizing effects on actin, both Hsp70 (Bidmon et al., 2000) and Hsp27 (Van Why et al., 2003) inhibit ATP depletion-mediated disruption of Na,K-ATPase, an actin linked transporter. Hsp70 complexes with denatured cellular proteins including Na,K-ATPase in an ATP-dependent manner and after ischemic stress, likely refolds and stabilizes Na,K-ATPase and other aggregated cytoskeletal components to facilitate their reassembly (Aufricht et al., 1998a). Importantly, HSPs expression failed to preserve ATP content levels in these studies (Van Why et al., 2003; Wang and Borkan, 1996), suggesting that protection by HSPs is not simply related to cell energy content. In addition to Hsp27 and Hsp70, Hsp90 expression is also induced by renal ischemia and likely operates in a synergistic fashion with these two HSPs to stabilize the interaction between Na,K-ATPase and the cytoskeleton (Bidmon et al., 2002).

RENAL INFLAMMATION AND HSP

Substantial evidence now suggests that HSPs, considered to act exclusively in the intracellular milieu as cytoprotectants, also signal impending danger to other cells

in the body when present in the extracellular compartment. In fact, "cytoprotective proteins" can become "molecules of destruction" when chaperones are expressed on the cell surface. The release of HSPs from cells occurs during cell death by necrosis or selectively via an unidentified secretory mechanism that does not require disruption of the plasma membrane (Bausero et al., 2005). Extracellular HSPs bind to the surface of adjacent and/or remote cells, increase cytokine transcription and act as pro-inflammatory molecules (Asea, 2008; Asea et al., 2000; Bausero et al., 2005). The presence of HSPs on the surface marks some cells for apoptosis (Poccia et al., 1996), whereas in others it promotes inflammation (Moseley, 2000; Trieb et al., 1996).

Extracellular HSPs are found in a variety of clinical scenarios associated with physiologic or pathologic stress (Guisasola et al., 2008; Wright et al., 2000). For example, exercise increases the circulating level of Hsp70 without overt, untoward consequences. Yet in some disease states, circulating HSPs levels correlate with disease severity. Specifically, a high serum Hsp70 level in patients with systemic sclerosis is associated with oxidative stress, inflammation, increased renal vascular resistance, pulmonary fibrosis and skin sclerosis. These results suggest that Hsp70 may be a useful marker of disease activity (Ogawa et al., 2008) and may be involved in disease progression. Current studies show that HSPs are important in both innate and acquired immune responses and could have clinical applications as immunomodulators. Both pro-inflammatory and regulatory HSP-reactive T cells have been described in animal models of autoimmune disease (Moseley, 2000). Indeed, elevated levels of anti-HSPs autoantibodies (e.g., anti-Hsp60, anti-Hsp70) have been described in adults with atherosclerotic lesions and cardiovascular events (Guisasola et al., 2008; Wright et al., 2000) in which inflammation is thought to be important.

Recent observations indicate that heat preconditioning sufficient to induce intracellular HSPs exerts an anti-inflammatory effect, perhaps by suppressing NFκB (Jo et al., 2006). The observation that NFκB activation after ischemia/reperfusion is suppressed by heat pre-conditioning and is associated with lower monocyte chemoattractant protein-1 expression and inflammatory cell infiltration support this hypothesis. Inhibition of Hsp70, the most abundant HSPs induced by heat preconditioning, reversed this functional protection. These data provide evidence that Hsp70 affords protection partly via inhibition of NFκB-mediated inflammation (Jo et al., 2006).

Chronic interstitial nephritis after heavy metal exposure is also associated with inflammation, largely driven by T lymphocyte activation in response to an immunodominant Hsp70 peptide. Passive transfer of Hsp70 reactive, kidney-derived T cells harvested from cadmium-exposed mice precipitated acute tubulo-interstitial nephritis in mice. In contrast, the passive transfer of control cells to the region beneath the renal capsule failed to induce nephritis in the recipient mice (Weiss et al., 1994). These results indicate that Hsp70 is a key target for T cell-mediated inflammation that together with Hsp70 reactivity causes tubular injury in this chronic model of toxin-induced nephritis.

Interestingly, selective up-regulation of Hsp70 using geranylgeranylacetone (GGA) inhibits pro-inflammatory cytokine liberation and nitric oxide (NO) production in lipopolysaccharide (LPS)-treated rats and most importantly, protects rats against death caused by LPS-induced endotoxin shock, an established model of sepsis. In this important study, protection by GGA correlated with Hsp70 induction in multiple organs and protection was inhibited by quercetin, an Hsp70 inhibitor (Nakada et al., 2005). This study suggests that manipulation of Hsp70 is a viable methodology for improving clinical outcomes in multisystem diseases.

ROLE OF HSP IN RENAL TRANSPLANTATION

Removal, storage, and re-implantation of a kidney are accompanied by a variable degree of ischemic damage (Perdrizet et al., 1993; Womer et al., 2000). Ischemic renal injury is an important cause of early graft failure (Dragun et al., 2000) and may increase the risk of subsequent acute or chronic rejection (Womer et al., 2000). Although therapy with high doses of immunosuppressants is often effective for acute allograft rejection associated with active inflammation, no treatment presently exists for chronic allograft nephropathy (CAN), a persistent state of low-grade rejection and inflammation characterized by tubular atrophy, interstitial fibrosis, glomerulosclerosis and progressive renal failure.

Several experimental transplant models suggest that up-regulation of renal HSPs is linked to renal allograft survival and function. In cell culture, cold storage followed by re-warming induced cell death associated with decreased expression of Hsp70, 90, and 27 as well as Bcl2, an anti-apoptotic protein. Sublethal heat stress reduced cell death caused by cold storage and re-warming and was associated with the preservation of Hsp70, Hsp27, and Bcl2 protein content (Healy et al., 2006). Surprisingly, preventing heat stress-induced Hsp70 up-regulation with siRNA did not significantly block the protective effect of heat stress against cold storage and re-warming cell death, suggesting either that Hsp70 may not be essential or that siRNA-mediated Hsp70 suppression was inadequate. The latter is suggested by the observation that selective Hsp70 expression afforded a similar level of protection as prior heat stress (Healy et al., 2006). Interestingly, pre-treatment with low-dose CsA or FK506 prevented subsequent ischemia-reperfusion injury in the rat, an effect that may be related to their ability to induce Hsp70 (Yang et al., 2001). Whether or not pretreatment of human renal donors with low-dose CsA or FK506 results in an improvement in post-transplant function is not yet known.

Despite substantial, but indirect evidence for the role of Hsp70 in allograft function, some contradictory evidence exists. Low levels of Hsp70 expression in kidney biopsies obtained from pre-transplant human donors failed to predict delayed graft function or acute rejection (Mueller et al., 2004). One unifying interpretation of these results is that both processes are multi-factorial and not necessarily related to organ ischemia. In addition to Hsp70, Hsp27 may also be involved in chronic allograft

nephropathy. Six months after transplantation, renal Hsp27 mRNA and protein levels were reduced and Hsp27 had "shifted" from the medulla to the cortex in rats with chronic allograft nephropathy (Djamali et al., 2005). The role of Hsp27 as a marker or mediator of chronic allograft rejection is presently unclear.

A novel Hsp (Hsp45 kDa or HJD-2) has been identified in human kidney biopsies with histologic evidence of either acute or chronic rejection. In contrast, both pretransplant kidneys and transplanted kidneys without evidence of acute or chronic rejection failed to express HJD-2 (Alevy et al., 1996). The investigators hypothesized that HJD-2 might be an antigen against which cytotoxic T cells that mediate acute rejection are directed. However, Hsp60 was also increased in allografts with rejection (Alevy et al., 1996), making it difficult to ascribe causality to a single HSPs. Furthermore, interstitial accumulation of Hsp47, an unrelated HSPs of similar molecular weight, positively correlates with the degree of interstitial fibrosis in allografts with chronic progressive dysfunction (Abe et al., 2000).

Recently, the role of extracellular HSPs in renal transplantation has also been investigated. Autoreactivity to Hsp60 and Hsp70 was analyzed by examining the proliferation of human peripheral mononuclear cells to purified HSP in the presence of exogenous cytokine before and after renal transplantation (Granja et al., 2004). Their results suggest that Hsp60 or Hsp70 in the extracellular environment regulates reactive T-cell proliferation, predisposing transplant patients to allograft rejection. Although the events that cause Hsp60 or Hsp70 release in these patients remain to be determined, HSPs represent a potential marker for allograft injury, a possible mediator of allograft ischemia and inflammation, and/or a therapeutic tool to improve allograft survival.

HSP AS ANTI-APOPTOTIC PROTEINS

Of the described mechanisms of cell death, the relationship between stress proteins and apoptosis after ischemia is one of the best characterized. Programmed apoptosis is a form of cell death required to remove unwanted or excessive cells during development and regulates normal adult homeostasis (Jacobson et al., 1997). In contrast, apoptosis also occurs as a result of catastrophic insults including autoimmune, inflammatory and neurodegenerative diseases, ischemia/reperfusion injury and cancer (Fadeel and Orrenius, 2005). The morphologic endpoints of apoptosis are organized DNA fragmentation, nuclear condensation, membrane blebbing, and "packaging" of the cytoplasm into distinctive "apoptotic bodies" followed by phagocytotic clearance by normal, surrounding cells (Fadeel and Orrenius, 2005; Wang et al., 1999). In the kidney, apoptosis has been implicated in glomerulonephritis, acute and chronic renal failure, obstructive uropathy, diabetic nephropathy, and polycystic kidney disease (Ortiz et al., 2001), suggesting that this often under-recognized cell death pathway likely contributes to organ dysfunction in these conditions. The link between specific HSPs and apoptosis has successfully illuminated both the role

of apoptosis in renal injury and the substantial anti-apoptotic effects of select HSPs including Hsp70 and Hsp27.

APOPTOSIS VERSUS NECROSIS IN ISCHEMIC ACUTE KIDNEY INJURY (AKI)

Most investigators now believe that apoptosis contributes to ischemic renal dysfunction (Bonegio and Lieberthal, 2002; Chiang-Ting et al., 2005; Jani et al., 2004; Kunduzova et al., 2003; Ortiz et al., 2003; Saikumar and Venkatachalam, 2003). Consistent with this hypothesis, several reports showed that targeted anti-apoptotic treatment (caspase inhibition, Bcl2 adenoviral delivery, erythropoietin) reduced apoptosis in the proximal tubule in the cortex and outer medulla and improved renal function after ischemia in the rodent (Chiang-Ting et al., 2005; Daemen et al., 1999; Faubel et al., 2004; Kunduzova et al., 2003; Xue et al., 2007). Evidence for apoptosis in the kidney has been confirmed using diverse approaches such as Hoechst staining, caspase 3 activation, cytochrome c release, TUNEL, Bax activation, loss of Bcl2 and ultimately by morphologic criteria using electron microscopy (Chiang-Ting et al., 2005; Kelly et al., 2003, 2004; Oberbauer et al., 2001; Spandou et al., 2006).

The mitochondrion represents a key site for integrating pro- and anti-apoptotic signals during stress. Ultimately, the balance between pro-and apoptotic forces determines whether or not the cells die by the intrinsic apoptotic pathway by regulating mitochondrial membrane permeability, the equivalent of opening "Pandora's Box" (Zamzami and Kroemer, 2001). BCL2 family proteins provide critical control over mitochondrial membrane permeabilization. Protecting the mitochondrial membrane is crucial, since stress-induced permeabilization permits the leakage of pro-apoptotic proteins (cytochrome c and AIF) that activate caspase-dependent and independent cell death pathways, respectively (reviewed in Chipuk et al., 2006; Er et al., 2006). Bax and Bak are the primary members of the BCL2 family that increase mitochondrial membrane permeability in renal epithelial cells, whereas Bcl-2 and Bcl-X_l antagonize Bax or Bak mediated membrane "attack." Both metabolic stress in vitro (Meldrum et al., 2001; Mikhailov et al., 2001; Oberbauer et al., 2001; Ortiz et al., 2000; Saikumar et al., 1998; Wang et al., 1999) and renal ischemia in vivo (Chiang-Ting et al., 2005; Chien et al., 2001; Ortiz et al., 2000; Schumer et al., 1992) increase the Bax:Bcl2 ratio in renal epithelial cells, a primary determinant of cell death (Korsmeyer, 1999). In renal cells, ischemia activates Bax (Mikhailov et al., 2003, 2001; Ruchalski et al., 2006) and reduces Bcl2 (Chien et al., 2001; Wolfs et al., 2005), markedly altering the Bax:Bcl2 ratio in a pro-apoptotic direction (Wang et al., 1999). Mitochondrial membrane injury has been attributed to other members of the BCL2 family including t-Bid, the active form of Bid (Wei et al., 2004). However, Bax-Bak double knockout cells (Danial and Korsmeyer, 2004) or mice (Lindsten et al., 2000), exhibit resistance to apoptotic stimuli including t-Bid (Wei et al., 2001), suggesting that Bax (perhaps in conjunction with Bak) is essential for mitochondrial

membrane permeabilization (Waterhouse et al., 2002). This conclusion is consistent with the recent observation that Bid knockout delays but does not prevent organ failure in mice subjected to renal ischemia (Wei et al., 2006). Stress signals may tip the balance in favor of pro-apoptotic BCL2 family members through phosphorylation, cleavage, or conformational change, and/or by releasing their anti-apoptotic family members (Fadeel et al., 1999; Yang and Korsmeyer, 1996). Which members of the family are affected depends upon the specific stress. After ischemia in vivo (Chiang-Ting et al., 2005) or metabolic stress in vitro (Havasi et al., 2008), Bax activation is well established.

Hsp70 has been implicated as a cytoprotectant in the ischemic kidney (Emami et al., 1991; Yang et al., 2003), and biochemical mechanism(s) that antagonize cell death have begun to be elucidated. Early reports emphasized the post-mitochondrial effects of Hsp70 on apoptosis (e.g., apoptosome assembly (Beere et al., 2000) and caspase 3 activation (Li et al., 2000)) but subsequent studies show that post-mitochondrial effects were caused by the buffer rather than Hsp70 itself (Ran et al., 2004; Steel et al., 2004). Mosser and colleagues showed that Hsp70 is unable to prevent cell death after mitochondrial disruption and caspase 3 activation, suggesting that Hsp70 inhibits apoptosis by protecting mitochondria (Stankiewicz et al., 2005). Recent observations, including those from our laboratory, confirm that Hsp70 prevents Bax-mediated mitochondrial membrane injury and allows pro-apoptotic signal proteins to be retained in the organelle's inter-membranous space (Creagh et al., 2000; Li et al., 2002; Ruchalski et al., 2006; Stankiewicz et al., 2005; Steel et al., 2004), supporting the hypothesis that Hsp70 acts as a "primary defense system" upstream of organelle injury. Primary protection is imperfect however, since some mitochondrial membrane injury occurs even after Hsp70 expression is enhanced to a level that equals or exceeds physiologic heat stress (Li et al., 2002; Ruchalski et al., 2003). In fact, modest AIF and cytochrome C leakage persist in renal cells with enhanced Hsp70 expression (Li et al., 2002). Fortunately, Hsp70 provides "secondary protection": by binding and sequestering both AIF (Ravagnan et al., 2001; Ruchalski et al., 2006) and cytochrome c (Li et al., 2002) that leak into the cytosol during stress.

Interestingly, distinct Hsp70 regions, including the carboxy-terminal chaperone (EEVD) and amino-terminal ATPase domain mediate primary and secondary cytoprotective effects. The EEVD domain that controls chaperone function is necessary to prevent mitochondrial membrane injury that results in the leakage of apoptosis inducing factor (AIF) whereas the ATPase domain is critical for sequestering cytosolic AIF thereby preventing nuclear AIF accumulation (Ruchalski et al., 2006). Similar domain specificity has been shown for Hsp70-mediated interaction with Bcl2 (Doong et al., 2002) and for cytoprotection in non-renal cells (Mosser et al., 2000). Together, these studies emphasize that HSPs act not only as classic molecular chaperones but also afford protection using non-chaperone domains that interact with established mediators of the apoptotic cell death pathway.

How Hsp70 inhibits Bax activation, resulting in less outer mitochondrial membrane injury and the nature of Bax-mediated membrane injury itself are the subject

of intensive investigation. Whether Hsp70 directly or indirectly inhibits Bax is critical for understanding its mechanism of action. Although Hsp70-Bax interaction has been reported by a single group (Gotoh et al., 2004), neither Mosser (Stankiewicz et al., 2005) nor our laboratory (Wang et al., 1999) detected such interaction using reciprocal co-immunoprecipitation under normal and pathophysiologic conditions that minimize non-specific binding. In addition, neither stress nor exogenous ATP modify Hsp70-Bax binding, conditions that differentiate specific from non-specific interactions between Hsp70 and its substrates (Aufricht et al., 1998b; Rauchman et al., 1997; Wang et al., 1999). Taken together, this suggests that Hsp70 inhibits Bax either by interacting with: (1) its regulatory proteins (including kinases such as Akt and GSK3β); (2) Bax co-factors or (3) Bax antagonists (e.g., Bcl2). At present, better understanding of the role of BCL2 proteins and Hsp70 is important if we are to improve the outcome of Bax-mediated renal injury.

In addition to Hsp70, Hsp90, Hsp60, and Hsp27 have been suggested to exert anti-apoptotic effects. In non-renal cells, Hsp27 prevents apoptotic stress signals such as oxidative stress, by increasing levels of intracellular glutathione, or aggregation of misfolded proteins, by acting as a molecular chaperone (Concannon et al., 2003). In renal cells, Hsp27 inhibits Bax activation, in part by preventing the loss of phosphatidyl inositide 3 kinase (PI3 kinase) activity responsible for activating Akt (Havasi et al., 2008). Presumably, Hsp27 inhibits stress-induced activation of Box by altering its phosphorylation by glycogen synthase kinase 3-beta (GSK3β) and/or by Akt. It has also been suggested that Hsp27's ability to stabilize actin filaments prevents pro-apoptotic proteins like Bax and Bid from translocating to mitochondria (Paul et al., 2002; Tang et al., 2006). F-actin reorganization following apoptotic stimuli leads to increased mitochondrial actin accumulation prior to Bax insertion or cytochrome c release (Tang et al., 2006). This actin-regulated effect may be involved in the translocation of pro-apoptotic proteins even without mitochondrial localizing sequence (e.g., Bax).

HSP AND OSMOLAR STRESS

Human renal inner medullary cells survive in a relatively hyperosmotic interstitium and depending on hydration state, face extracellular osmolarities ranging from 50 to 1200 mOsm. In fact, these epithelial cells withstand higher interstitial levels of NaCl and urea than anyother cell type. This osmotic stress is sufficient to alter function, decrease the proliferation rate and even induce death in cultured cells in proportion to the increase in NaCl content (Michea et al., 2000). In murine inner medullary collecting duct cells in culture and intact animals in vivo, acute increases in osmolality using NaCl cause DNA double strand breaks (Kultz and Chakravarty, 2001) that are not repaired as long as NaCl levels remain high, despite the fact that the cells have re-entered their normal cycle (Dmitrieva et al., 2003, 2004). In addition, elevations in NaCl and urea cause oxidative stress characterized by the formation of reactive oxygen species that precipitate 8-oxoguanine lesions (Greenberg, 2004;

Zhang et al., 2004) and protein carbonylation (Levine et al., 2000). Carbonylated proteins are usually not repaired but undergo proteasomal degradation (Levine, 2002). Relatively high levels of carbonylated proteins are found in inner medullary cells of the normal kidney and are indicative of chronic osmotic stress (Zhang et al., 2004). Although osmotic stress reduces the transcription and translation of most proteins (reviewed in Burg et al., 2007) that of specific HSPs increase.

When the level of hypertonicity reaches a threshold, renal epithelial cells undergo apoptosis. Evidence supports both the extrinsic (i.e., extracellularly regulated; Franco et al., 2002; Lang et al., 2002; Rosette and Karin, 1996) and intrinsic (i.e., mitochondrial; Michea et al., 2002) apoptotic pathways in this process. An interesting connection between hypertonicity and apoptosis may be the apoptotic volume decrease (AVD). AVD is typically iso-osmotic cell shrinkage that precedes cytochrome c release and is an integral part of the apoptotic process (Bortner and Cidlowski, 1998; Maeno et al., 2000). In fact, inhibiting AVD interrupts the apoptotic process (Bortner and Cidlowski, 1998; Maeno et al., 2000; Okada et al., 2001). Since cell shrinkage is one of the first consequences of hypertonicity, changes in cell volume per se could contribute to apoptosis. The normal cellular response to hypertonic shrinkage is termed the "regulatory volume increase" (RVI) in which electrolytes and water enter the cell to restore its volume. RVI is accomplished by activating ion carriers (NKCC1) and ion exchangers (NHEs) (Wehner et al., 2003). Cells with inhibited RVI or that lack a robust RVI, undergo apoptosis at much lower levels of hypertonicity (Bortner and Cidlowski, 1996). These observations implicate cell volume as a participant in the apoptotic signal cascade in renal epithelial cells.

Increased levels of heat shock proteins are detected as one moves along the corticomedullary gradient of the kidney and provide one mechanism for protecting cells in this ever-changing environment. Hypertonic stimulation induces HSPs within hours and counters the effect of increased ionic strength, presumably to reduce protein unfolding and aggregation (Cohen et al., 1991; Petronini et al., 1993). Furthermore, HSPs may also prevent cells from undergoing apoptosis during hypertonic stress (Santos et al., 1998b). Precisely how osmotic stress induces HSPs is unclear. Classic heat stress causes the monomeric heat shock factor 1 (HSF1) to trimerize, become phosphorylated, translocate to the nucleus, bind DNA, and become transciptionally competent (Baler et al., 1993; Sarge et al., 1993; Zuo et al., 1995), bind to heat shock elements (HSE) and thereby increase HSP transcription. However, some stressors, including hypertonic shock (Alfieri et al., 1996; Caruccio et al., 1997), cause trimerization, activation, and binding of HSF1, but fail to increase Hsp70 transcription (Bruce et al., 1993; Hensold et al., 1990; Jurivich et al., 1992). Despite this apparent contradiction, more prolonged (i.e., hours) exposure to a more modest degree of hypertonicity increased Hsp70 mRNA and protein content (Cohen et al., 1991; Neuhofer et al., 1999; Petronini et al., 1993) even in the absence of 3T3 HSF1 activation and Hsp70 gene transcription (Alfieri et al., 2002). These results suggest that osmotic stress may increase Hsp70 content by stabilizing its mRNA (Alfieri et al., 2002).

Others suggest that transcription factors besides HSF1 mediate the effects of hypertonicity on Hsp70 expression. For example, hypertonicity stimulates the transcription of tonicity-responsive enhancer binding protein (TonEBP), resulting in increased organic osmolyte content inside the cell. TonEBP also stimulates vasopressin-regulated urea transporter (UT-A), a factor that contributes to the high concentration of urea in the renal medulla. In fact, the Hsp70-2 isoform has three osmotic response element (ORE) sites that respond to TonEBP binding (Woo et al., 2002) and its transcription is increased by binding to TonEBP in response to hypertonicity (Heo et al., 2006). Hsp70 has been shown to protect cells against high levels of urea (Neuhofer et al., 2001; Santos et al., 1998a), whereas Hsp70 knockdown increases urea-induced cell death (Neuhofer et al., 1999). TonEBP, responsive to extracellular urea-mediated osmotic stress, also appears to enhance Hsp70 expression to protect against the urea. The exact mechanism by which Hsp70 and the other stress proteins (e.g., HSP 25, 60, 110, 200 and the osmotic stress protein OSP94) protect against osmotic stress is unclear (Beck et al., 2000; Borkan and Gullans, 2002). It is apparent however, that Hsp70 is crucial for preventing injury caused by osmotic stress, since hypertonicity causes renal cell death after knockout of HSP70.1 (the mouse homolog to the hypertonicity sensitive human HSP70-2) in vitro (Lee et al., 2005; Shim et al., 2002) and in intact mice in vivo (Shim et al., 2002).

CONCLUSION

There is an intimate relationship between physiologic stress and classic stress protein response in the kidney under normal and pathophysiologic conditions. Using stress proteins as "detective tools," investigators have begun to identify many of the lethal and sublethal signal events that regulate renal cell injury caused by ischemia, hypoxia, inflammation and nephrotoxin exposure. Despite the established protective effects of HSP, practical strategies for up-regulating human HSP remain elusive. Hsp90-binding agents geldanamycin and its analogs (17-AAG and 17-DMAG), increase the expression of protective HSP. Partly by reducing oxidative stress, these HSP increase the resistance of renal adenocarcinoma cells in vitro to injury caused by chemotherapeutic agents and in vivo, reduce ischemia-reperfusion injury in the mouse kidney (Harrison et al., 2008). Similarly, induction of Hsp70 by geronylgeronlyacetone (GGA), well tolerated in humans with peptic ulcer disease, attenuates ischemic renal injury in rats when given before the insult (Suzuki et al., 2005). These preliminary findings support the contention that pharmacologic or molecular-induced up-regulation of endogenous HSP will improve renal outcomes for patients subjected to a stress sufficient to cause organ injury.

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CHAPTER 4

HEAT SHOCK PROTEIN AND INFLAMMATION

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Abstract: Heat Shock Proteins (HSP) are important modulators of both anti-inflammatory and pro-inflammatory responses. In this chapter, we address this apparent paradox by focusing on the effects of the highly heat inducible Hsp70 and its transcription machinery. This transcription machinery may exert important effects on inflammation through pathways, which are independent of heat shock proteins. We then discuss disease states where the balance between the anti-inflammatory and pro-inflammatory effectors is critical to disease outcome

Keywords: Cytokines; HSF-1; HSP; inflammatory response syndrome; heat stroke; inflammation; heat injury

Abbreviations: AP-1, activator protein 1; APC, antigen presenting cells; eHSP, extracellular HSP; GLN, glutamine; HMGB1, high-mobility group box 1 protein; HIV, human immunodeficiency virus; HSF-1, heat shock transcription factor 1; HSP, heat shock protein; HSR, heat shock response; IBD, inflammatory bowel diseases; IFN-γ, interferon-gamma; IKK, IkappaB kinase; IL-6, interleukin 6; JNK, c jun amino terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; MODS, multiple organ dysfunction syndrome; NF-κB, nuclear factor kappa B; NF-IL6, nuclear factor-IL6; NK, natural killer cells; SIRS, systemic inflammatory response syndrome; TLR, Toll like receptor; TNF-α, tumor necrosis factor alpha

INTRODUCTION

The heat shock response (HSR) is one of the most evolutionary conserved protective mechanisms in cells. It involves temporary modifications in gene expression and synthesis of different heat shock protein (HSP) family members to help the organism to cope with environmental and physiological stresses. In non-stressed conditions, HSP function as molecular chaperones, maintaining protein conformation and facilitating transport. In response to stress, HSP expression prevents protein aggregation, refolds

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damaged proteins, and promotes the degradation of irreversibly denatured proteins. While the cellular protein management functions of HSP are well described, this chapter focuses on the ability of the HSR to modulate the immune/inflammatory responses of multicellular organisms. In this regard, the components of the HSR, including HSP, especially the most highly heat inducible member, heat shock protein 70 (Hsp70), and heat shock transcription factor 1 (HSF-1) have evolved to act as both pro- and anti-inflammatory molecules in the production and release of inflammatory modulators. As an anti-inflammatory effector, the HSR modulates cytokine signal transduction and gene expression by inhibiting the translocation of the transcription factor nuclear factor-kappa B (NF-κB) to the nucleus and preventing the expression of inflammatory mediators. As pro-inflammatory mediators, necrotic and non-necrotic release of HSP into the extracellular environment produces a multifaceted immune/inflammatory response involving the activation of a variety of immune effector cells and cytokine release. Finally, the crucial indirect role of the HSR in maintaining gut epithelial barrier integrity has important anti-inflammatory effects on the movement of endotoxin into the circulation, and so helps to prevent the subsequent endotoxin-mediated inflammatory cascade. Therefore, the ability of the HSR to regulate inflammation is, in turn, an important aspect of the progression of a variety of pathophysiologic states such as the sepsis and heat stroke, which are characterized by dysregulated inflammatory response and multiorgan-dysfunction. In this chapter, we discuss the role of the HSR in activating both anti- and pro-inflammatory systems in the whole organism, the differences seen between human and non-human model systems, potential mechanisms by which the HSR could exert its effects on inflammation, and disease states where the HSR may alter pathophysiology. We have chosen to focus on a fairly linear model comprising the activation of HSF-1 and Hsp70. While other HSP such as Hsp27, the endoplasmic reticulum chaperone gp96, bacterial Hsp60, and others have been associated with immune and autoimmune disease states, the highly inducible Hsp70 provides a useful platform to discuss the role of these primitive and ubiquitous proteins in modulating human inflammatory disease.

HSP ARE ANTI-INFLAMMATORY

Initial observations in animals linked the HSR to an altered inflammatory response through the demonstration that heat conditioning conferred survival to an otherwise lethal endotoxin stress (Ryan et al. 1992). Subsequent studies demonstrated that this increased survival in endotoxin exposed animals was associated with elevated levels of intracellular Hsp70 in the liver as well as lower serum concentrations of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) but not interleukin 6 (IL-6; Kluger et al. 1997). Parallel data in cell culture demonstrated that heat conditioned peritoneal macrophages showed a decrease in endotoxin induced TNF-α transcription and secretion which was sustained for as long as the cells had elevated Hsp70 levels (Snyder et al. 1992, Ensor et al. 1994). Similar to the studies in the intact organism, endotoxin induction of IL-6 was unchanged in the heat conditioned cells. Interestingly, the decrease in cytokine production was also associated with a

decrease in cytokine mRNA suggesting an alteration on the transcription factor that regulates cytokine transcription genes.

The liver has been a convenient tissue to measure the HSR in animals because of the massive accumulation of Hsp70 following heat stress (Flanagan et al. 1995). While the large induction of the Hsp70 in liver has been a useful biomarker of stress, there is compelling data to support the direct link between liver Hsp70 accumulation and the altered survival and inflammatory cytokine profile seen in heat conditioned animals undergoing endotoxin stress. For example, when animals are treated with the liver protein synthesis inhibitor, D-galactosamine, to block liver HSP accumulation during the same heat conditioning regimen shown to induce endotoxin tolerance and decrease serum TNF-α, the result is an augmentation of endotoxin end organ damage and a marked increase in endotoxin induced TNF-α even though all organs but the liver showed the characteristic Hsp70 accumulation from the heat conditioning (Dokladny et al. 2001). These data demonstrate the importance of heat associated protein synthesis in the liver in conferring the altered inflammatory response and altered survival seen in endotoxin exposure in animals.

Heat is a pleotrophic stimulus, which activates numerous tissue responses altering not only the HSR but other protective systems important during endotoxin shock. The role of the HSR in modulating inflammation has been explored at several levels. Using a whole animal model, Xiao et al. (1999) demonstrated that mice deficient in HSF-1 are unable to generate a HSR, showing reduced survival and excessive production of TNF-α. At the protein level, studies of single gene over-expression have demonstrated that the protective effects of heat conditioning on subsequent endotoxin exposure can be conferred by Hsp70 expression alone in cells and in the whole organism. In this regard, Lau et al. (2000) used hearts of transgenic mice over-expressing Hsp70 to demonstrate a direct relationship between the level of Hsp70 expression and endotoxin tolerance. Ding et al. (2001) also reported that human peripheral blood monocyte-derived macrophages over-expressing Hsp70 inhibited Lipopolysaccharide (LPS)-induced production of TNF-α, IL-1β, IL-10 and IL-12. Recently, we have employed an adenovirus expression system to direct the expression of Hsp70 in the liver of rats. Liver Hsp70 expression duplicated the protective effects of whole body heat-conditioning by inhibiting cytokine production (TNF-α and IL-6) after endotoxin challenge (Dokladny et al. 2009). These data taken as a whole, demonstrate that Hsp70 expression is sufficient to alter pro-inflammatory cytokine production, increase endotoxin tolerance and survival in both cellular and intact organism and further suggest that specific organs such as the liver may be important for the effects of the HSR.

HSP Induction: How do HSP Directly Alter Cytokines?

Immune cells are able to detect low levels of LPS through its binding to toll-like receptors and initiate an inflammatory response by the activation of signaling pathways and transcription factors. The activation of various mitogen-activated protein kinases (MAPK) and IkappaB kinase (IKK) pathways directly or indirectly

phosphorylate/activate different transcription factors, including NFκB, nuclear factor interleukin 6 (NF-IL6), and activator protein 1 (AP-1). The NFκB transcription factors play a pivotal role in many cellular processes altering the expression of cytokines, chemokines, cell adhesion molecules, growth factors, anti-apoptotic proteins, and immunoreceptors (reviewed by Brasier 2006). Inactive NFκB is normally found in the cytoplasm bound to its inhibitory protein, IkB. NFκB is activated by a number of incoming signals from the cell surface, including ischemia, oxidative stress, and LPS. These signals lead to activation of IKK, which phosphorylates IkB, allowing NFκB to translocate into the nucleus and bind to its target gene (Zhang and Ghosh 2000). The targeted genes include those that activate inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-12.

A number of potential explanations have been suggested for the HSP mediated NFκB repression. There are data from cell culture and intact animal studies supporting direct physical interactions between Hsp70 and the NFκB inhibitor protein, IkB-α, which appear to prevent NFκB dissociation (Wong et al. 1997, Malhotra and Wong 2002). Sun et al. (2005) demonstrated that LPS exposure – induced NFκB activation was suppressed by Hsp70 accumulation (induced by sodium arsenite) with a subsequent decrease in TNF-α production and mRNA expression. Additionally, Hsp70 blocked IκB-α degradation and up-regulated IκB-α mRNA expression. Using intact animals, Pritts et al. (2000) showed that either heat exposure or sodium arsenite infusion inhibited increases on NFκB activity and decrease in IκB-α after induction of the stress response in vivo decreases NFκB activity in jejunal mucosa of endotoxemic mice. Another possibly mechanism is Hsp70 indirect mechanisms, whereby repression of MAPK activation mediates the inhibition of NFκB cascade. In addition, Hsp70 related activation of Jun kinase (JNK) MAPK induces phosphorylation of c-JUN (combination with c-Fos) to activate the transcription factor AP-1. The AP-1 is involved in the up-regulation of the IL-18 pro-inflammatory cytokine, for which overproduction is related to severe inflammatory disorders. Wang et al. (2002) observed that HSR could suppress the expression of IL-18 in response to LPS through inhibition of the JNK MAPK signaling pathway. Recent identification of a novel inflammatory cytokine and late inflammatory mediator of endotoxin lethality, high-mobility group box 1 protein (HMGB1), has provided further insights into another potential site of action of Hsp70 in modulating the inflammatory response. It has been shown that HMGB1 can trigger the MAPK pathway and subsequent NFκB transcription factor also stimulating the synthesis and release of pro-inflammatory cytokines. Tang et al. (2005) observed that murine macrophage-like RAW264.7 cells, stimulated with LPS released HMGB1, induced the release of other pro-inflammatory mediators and mediated lethality. However, a heat shock pretreatment suppressed the release and translocation of HMGB1. Therefore, induction of Hsp70 by heat shock, sodium arsenite or over-expression can be an important factor in reducing mortality in experimental models of septic shock by down-regulating expression of inflammatory genes, such as TNF-α, IL-1, IL-12, and IL-18. These studies demonstrate a great degree of redundancy in Hsp70 cytokine interactions and are summarized in Figure 1.

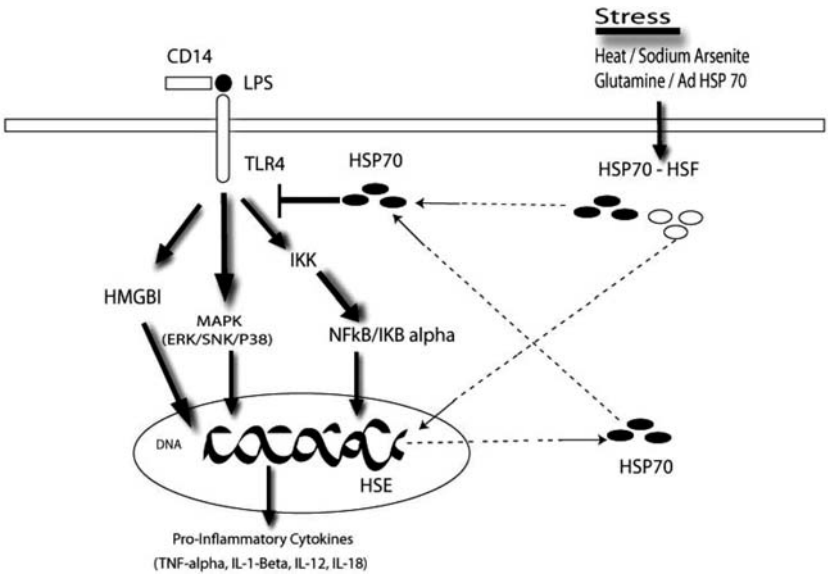

Figure 1. Interaction between HSP and cytokine signal transduction pathways

Are HSP Involved in Muscle IL-6 Production?

One of the interesting paradoxes of Hsp70 associated cytokine gene regulation is the concordant activation of both Hsp70 and IL-6 in exercising muscle. The large increase in serum IL-6 following exercise has been demonstrated to be of muscle origin (Pedersen et al. 2001). This IL-6 response appears to be distinct from endotoxin associated IL-6 production as described above. Further distinguishing the IL-6 response to exercise from that driven by endotoxin is its association with HSF-1 activation and cellular Hsp70 accumulation. As described above, cellular Hsp70 accumulation and HSF-1 activation both exert a potent inhibitory effect on NFκB mediated inflammatory signaling pathways. In contrast, HSF-1 activation and muscle cell Hsp70 accumulation precede muscle cell IL-6 production. Further, agents such as glutamine, which activate HSF-1, and which are associated with an HSP dependent inhibition of inflammatory IL-6 actually augment exercise induction of IL-6 (Hiscock et al. 2003). These data suggest a link between HSR and exercise associated IL-6 production in muscle, in contrast to the potent HSR inhibitory effects of inflammation.

HSF-1 as Non-HSP Transcription Factor Influences Inflammation

Hsp70 alone, whether induced as a part of the HSR or through the use of single gene expression systems, is sufficient to inhibit the production of cytokines in response to an inflammatory stimulus such as LPS. There is also data demonstrating that

the HSR mediates cytokine expression through direct actions of HSF-1 distinct and independent from cellular HSP accumulation. Cahill et al. (1996) first demonstrated that HSF-1 binds to the IL-1β promoter and represses its activity. Using HSF-1 knockout mice ($HSF^{1-/-}$), Wirth et al. (2004) also observed higher inflammatory mediators and severe lung damage compared to wild type mice after cadmium exposure. It was also reported that DNA binding of the transcription factor NFκB was higher in $HSF^{1-/-}$ mice than in wild-type mice after cadmium exposure. Previous study has also observed that over-expression of HSF-1 can increase the expression of anti-inflammatory IL-10 (Xiao et al. 2006). Specifically to TNF-α, Singh et al. (2002) identified a binding site in the TNF-α promoter for HSF-1 which represses the transcription of TNF-α. However, the anti-inflammatory activity of HSF-1 is not limited to its effects on cytokine transcription. In fact, recent data demonstrate that HSF-1 serves as a transcription factor for the gut tight junction protein, occludin-1 (Dokladny et al. 2008). The ability of HSF-1 to induce occludin-1 transcription may be an important part of the well-described ability of heat conditioning to preserve epithelial barrier integrity during heat stress (Moseley 1994). This increased epithelial barrier resistance may be a key factor in limiting gut associated endotoxin translocation during heat stress, and low perfusion states. Several inflammatory states are associated with loss of the intestinal barrier resulting in translocation of endotoxin, which enhances inflammation and leads to systemic inflammatory response syndrome (SIRS). Thus, in the intact organism, HSF-1 activation may limit endotoxin translocation through transcription of tight junction proteins, thereby limiting inflammation in an indirect fashion.

Finally, HSF-1 may also be linked with inflammatory pathway transduction and apoptotic cell signaling. Recently, Franceschelli et al. (2008) showed that Bag3, a protein with anti-apoptotic role, is regulated by HSF-1. Down-regulation of HSF-1 reduced the Bag3 protein levels, and increased the incidence of cell apoptosis. Additionally, Chen and Currie (2006) also demonstrated that knocking down HSF-1 gene caused significantly higher activation of NFκB and the pro-apoptotic protease activating factor 1 (Apaf-1), corroborating that HSF-1 has a more general role than specific cytokine regulation. Although these studies indicated that HSF-1 controlled these regulatory pathways we can not completely exclude the participation of HSP, since they also are known for the regulation of signal transduction and cellular survival pathways.

Glutamine (GLN) as HSF-1 Inducer

GLN provides an interesting opportunity to study the impact of nutrition and its potential role in activating the HSR as well as providing a model of the complex roles of HSR activators in altering the inflammatory response. There is evidence suggesting a link between GLN supplementation, activation of the HSR, and improved outcome of inflammatory conditions, such as those observed in sepsis, trauma, burns, and some critical illness (Griffiths et al. 1997, 2002, Morlion et al. 1998,

Novak et al. 2002, Wischmeyer et al. 2001c, Oudemans-van Straaten et al. 2001). In these investigations, GLN supplementation has been associated with lower mortality, decreased infection, and shortened hospital stay in acute and/or critically ill patients. Conversely, the state of GLN depletion was shown to be an independent predictor of outcome in acute and/or critically ill patients (Oudemans-van Straaten et al. 2001, Roth et al. 1982). Parallel studies in various animal models of sepsis and severe injury demonstrated that GLN supplementation reduced tissue injury, inhibited pro-inflammatory response, improved survival, and exerted an important benefit in preventing gut-origin sepsis after trauma (Wischmeyer et al. 2001a, b, Gianotti et al. 1995).

There are several potential mechanisms by which GLN could be linked to improved outcomes in critical illness, sepsis and multi-organ dysfunction syndrome (MODS). One simple mechanism is that during severe illness the circulating levels of GLN fall abruptly (Greig et al. 1996) and GLN replacement is necessary to maintain the metabolic needs of immune cells such as lymphocytes, macrophages, and enterocytes (Spittler et al. 1995, Exner et al. 2003). This fall is especially true in healthy humans exposed to bacterial endotoxin (Vesali et al. 2005) but is, interestingly, also associated with the infusion of recombinant human IL-6 (rhIL-6; Van Hall et al. 2008). In this human study, there was a profound fall in serum GLN with a concomitant flux of GLN from muscle. Another interesting facet of GLN effects on cytokines is data from exercising muscle where IL-6 serves as a component of the anti-inflammatory myokine group rather than an LPS induced pro-inflammatory cytokine (Hiscock et al. 2003). In contrast to the inhibition of GLN on inflammation associated IL-6, GLN supplemented during exercise further elevates plasma levels of IL-6 compared to exercise alone.

Another potential mechanism for GLN associated protection is its ability to induce the HSR (Ehrenfried et al. 1995). In cell culture experiments, cells deprived of GLN show a decrease in heat induced Hsp70 and increased susceptibility to apoptosis, as well as reduced responsiveness to pro-inflammatory stimuli (Eliasen et al. 2006). Conversely, intestinal epithelial cells supplemented with GLN showed enhanced Hsp70 expression and were more resistant to thermal and oxidant injury (Wischmeyer et al. 1997). In animal models, intravenous GLN supplementation induces Hsp70 in multiple organs such as lung, kidney, heart and colon, and reduces mortality as well as end-organ injury after endotoxemic shock (Wischmeyer et al. 2001a, b). Previous research reported that increases in Hsp70 with GLN supplementation were accompanied by suppressed NFκB activation and inhibition of p38 MAPK, and ERK phosphorylation. Further, lower pro-inflammatory cytokine release (TNF-α and IL-1β) and oxidative stress were observed in GLN supplemented animals exposed to endotoxin (Singleton et al. 2005a). More recent studies suggest that the HSR is necessary for the blunted cytokine response and protection afforded by GLN (Morrison et al. 2006, Singleton and Wischmeyer 2007). The initial study by Singleton et al. (2005b) observed that a single-dose of GLN post-sepsis initiation increased lung Hsp70 and HSF-1, and improved survival. Interestingly, the beneficial effects of GLN on survival were lost after the administration of the Hsp70

blocker quercetin. Similarly, Morrison et al. (2006) demonstrated that mouse fibroblasts lacking *hsp*70 genes (HSF-1$^{-/-}$) but supplemented with GLN had decreased survival compared to wild type cells supplemented with GLN. Moreover, the same group confirmed these initial results using an intact animal model (Singleton and Wischmeyer 2007). They reported that mice supplemented with GLN but also lacking the *hsp*70 genes (HSF-1$^{-/-}$) show greater mortality, higher pro-inflammatory cytokine expression and higher NFκB activation. The authors concluded that Hsp70 expression is required for GLN's effects on survival, tissue injury, and the inflammatory response after sepsis. These data strongly support a role for HSF-1 activation and perhaps Hsp70 in GLN associated protection in animal models.

In contrast to the relatively clear support for GLN activation of the HSR in cell culture and animal studies, clinical trials in humans are much less clear. Cellular Hsp70 accumulation has been shown to be impaired in granulocytes, and lymphocytes of patients with polytrauma (Weingartmann et al. 1999, Schroeder et al. 1999). However, a double-blind trial observed that patients in the intensive care unit receiving GLN showed enhanced serum Hsp70 (Ziegler et al. 2005). This serum Hsp70 increase also correlated with improved clinical outcome. However, the function of serum Hsp70 in this improved survival is unclear. It may provide a convenient and easily measurable marker of HSF-1 activation or Hsp70 expression in other tissues or may itself play a role in the observed outcome. Recently, we were unable to detect an effect of GLN infusion on either intracellular or serum Hsp70 in the LPS-induced cytokine response in healthy humans (Dokladny et al. 2009).

GLN associated HSR mediated protection may not rely solely on Hsp70. Although the previous cell and animal studies have confirmed that HSR is necessary to GLN protection, it is less clear if Hsp70 is the effector or whether the requirement for HSF-1 activation mediates the GLN effects through non-Hsp70 pathways. As mentioned above, the importance of the HSR in mediating these effects is largely based on studies using engineered HSF-1$^{-/-}$ knockout systems (Peng et al. 2006, Singleton and Wischmeyer 2007). In these studies, the protective effect of GLN is lost without HSF-1 activation. While Hsp70 can suppress the activation and nuclear translocation of NFκB (Ran et al. 2004) and its related cytokines, attenuation of TNF-α occurs prior to an increase in Hsp70 protein detection, indicating that HSP translation might not be necessary to attenuate the TNF-α expression. In fact, Eliasen et al. (2006) showed that human monocytes deprived of GLN showed normal capacity of HSF-1 to bind to HSE and normal increases in Hsp70 mRNA, however the Hsp70 mRNA showed lowered stability compared to cells with GLN.

HSF-1 itself can attenuate the expression of pro-inflammatory cytokines by binding to HSE and blocking IL-1β (Cahill et al. 1996) and blocking NFκB activation or inducing transcription of non-HSP genes, such Occludin-1 (Dokladny et al. 2008). This induction of Occludin-1 provides a mechanism for improved epithelial barrier integrity following HSF-1 activation, which would not require Hsp70 expression. Thus, studies using HSF-1 knockout systems do not provide

evidence to exclude these other, non-HSP effects of HSF-1 in mediating GLN related alterations.

Another possible mechanism is that GLN can reduce the inflammatory response in whole-body by activating the HSR and increasing maintenance of the mucosal barrier following injury or illness (De-Souza and Greene 2005). LPS translocation affects the function and integrity of remote organs and tissues, causing SIRS and MODS. It has been shown that gut is affected by prolonged periods of physiologic stress characteristic of severe burns, sepsis, and trauma (Deitch 1992). GLN may play a protective role in the gut because of its role as a preferential substrate, maintaining the selective barrier permeability and function against unwarranted bacterial translocation. However, maintenance of the mucosal barrier with GLN supplementation may also occur through an up-regulated HSR. Gut Hsp70 up-regulation induced by GLN supplementation correlates with a decrease in intestinal permeability and plasma endotoxin (Singleton and Wischmeyer 2006). Hsp70 accumulation in epithelial cells maintains the intestinal permeability during heat stress (Moseley et al. 1994). As noted above, the preservation of epithelial barrier integrity associated with heat conditioning may also involve the up-regulation of occludin-1 (Dokladny et al. 2008). Therefore, GLN supplementation may preserve cell and organ function via the induction of the HSR decreasing intestinal permeability and plasma endotoxin, and consequently reducing inflammatory response.

Finally, GLN protective effects may be related in part to its role as a precursor of glutathione (GSH), and reducing oxidative stress. GSH is an antioxidant and plays a critical role in reducing free radicals and maintaining cellular redox status. Several clinical conditions are associated with reduced GSH levels, which can result a lowered cellular redox potential (Exner et al. 2000). Xue et al. 2008 observed that GLN supplementation reduced chemotherapy side effects in rats associated with increased in Hsp70 and lower oxidative stress, as measured by the ratio of rGSH:oxidized GSH in the colon. Moreover, epithelial cells over-expressing Hsp70 showed a greater ability to preserve GSH and consequently create an intracellular environment with increased reducing capacity in the face of hypoxia or ischemia (Guo et al. 2007). Thus, GLN may improve tolerance to oxidative stress, either by contributing to the HSR or by serving as a substrate for GHS. Conversely the increase in Hsp70 appears to preserve GSH and so could reduce the need for GLN consumption for GSH generation.

Taken as a whole, GLN provides an interesting probe for the study of the HSR in human inflammatory illnesses. Mechanistic studies in animals link GLN activation of HSF-1 as the critical component of its anti-inflammatory response, and GLN supplementation in admitted heterogeneous populations of critically ill humans has been associated with improved outcomes. At the same time, GLN has important non-HSP biologic effects; in addition, HSF-1's role as a transcription factor of non-heat shock genes provides alternative mechanisms for the potential role of GLN in altering the course of human inflammatory illnesses.

HSP ALTER CYTOKINE TOLERANCE

In addition to the ability to block cytokine production by immune effector cells, HSP also alters target cell tolerance to inflammatory cytokines exposure. Heat conditioning renders tumor cells resistant to TNF-α mediated cytotoxicity (Kusher et al. 1990, Gromkowski et al. 1989). Jaattela and Wissing (1993) confirmed that Hsp70, but not HSP27, protects highly TNF-sensitive murine fibrosarcoma cells from cytotoxicity mediated by TNF-α, using cells transfected with Hsp70. Additionally, Jaattela (1993) demonstrated that Hsp70 over-expression or inhibition of endogenous Hsp70 synthesis did not change the ability of tumor cells to bind TNF-α or internalize and degrade the receptor-bound TNF-α. However, the authors observed that both Hsp70-mediated and TNF-induced TNF resistance appears to be associated with impaired TNF-α induce phospholipase A2 activation.

The mechanisms by which the HSP confer protection from cytokines are not clear but may involve the interplay of intracellular signals related to cell survival, stress tolerance and inflammation. For example, it is well recognized that exposure of cells to TNF-α may induce apoptosis, through either the caspase cascade; or activation of two important transcription factors involved in the inflammatory response, AP-1 and NFκB (reviewed by Baud and Karin 2001). As described in the previous sections, the HSR has an array of protective properties, and thus can regulate the same apoptotic pathways and transcriptions factors associated with cytokine activation. Schett et al. (2003) reported that exposure of U937 macrophages to TNF-α caused transient downregulation of HSF-1 activation and Hsp70 synthesis, leading to cell apoptosis. Paradoxically, acute cytokine exposure (TNF-α or IL-1β) induced Hsp70 syntheses in cardiac myocytes and pancreatic islets (Sharma et al. 1996, Nakano et al. 1996, Helqvist et al. 1991). Moreover, Ran and Lu (2004) demonstrated that TNF-α triggered apoptosis was initiated when Hsp70 expression increased to high levels to disrupt NFκB signaling. Therefore, it seems that there is a tissue or cell specific response to cytokine exposure and a complex interaction among Hsp70, NFκB and apoptotic signals.

HSP70 AND GUT PERMEABILITY

In addition to nutrient digestion and absorption, the gut functions as a barrier to prevent the translocation of intraluminal bacteria endotoxin into the portal circulation and systemic circulation. However, under some clinical conditions such as burn, trauma and critical illness (Ziegler et al. 1988, Langkamp-Henken et al. 1995), the gut barrier integrity is compromised and may result in the translocation of bacterial endotoxin. The mechanisms underlying gut barrier dysfunction in these conditions are not completely understood, but are at least temporally related to splanchnic hypoperfusion, lack of enteral feeding and associated gut ischemia (Maynard et al. 1993). Although, increases in gut permeability, followed by endotoxin translocation to the systemic circulation are frequently observed in animal experimental models,

these are not consistently reported in clinical studies in humans (Moore et al. 1991). Newer evidence suggest that shock, trauma, or burn-induced gut injury can cause local gut and gut-associated lymphatic tissues to produce cytokines and other inflammatory mediators, which may lead to lung, kidney, and liver dysfunction associated with sepsis and SIRS (Deitch 2002). The authors also provide an alternative route for the inflammatory products whereby bacterial translocation across the gut cause local activation of the gut inflammatory response reaching the systemic circulation not via the portal circulation but via mesenteric lymph. Thus, it seems that the loss of intestinal permeability drives the systemic inflammatory response and the multi organ dysfunction which occur in injured and critically ill patients.

The ability to maintain gut barrier function during critical illness as well as during splanchnic hypofusion states, such as elevated temperature with extreme skeletal muscle work, may be important in preventing systemic inflammation (Moseley 1994, Dokladny et al. 2006). In this regard, the induction of the HSR clearly attenuates the loss of epithelial barrier and function, both through the cellular accumulation of Hsp70 and through the HSF-1 up-regulation of transmembrane tight junction proteins, occludin-1 (Dokladny et al. 2006, 2008). Studies using epithelial monolayers cells demonstrate that Hsp70 accumulation driven by a non-lethal heat challenge or through genetic modification prevent the loss of epithelial barrier integrity to a lethal heat shock (Moseley et al. 1994, Dokladny et al. 2006). Additionally, Eaves-Pyles et al. (2000) have demonstrated that mice treated with sodium arsenite to induce cellular Hsp70 accumulation show increased survival and decreased bacterial translocation in a mouse burn injury model. Although these previous studies link Hsp70 to improved epithelial barrier tolerance, the recent report that heat stress induces the expression of occludin-1 by HSF-1 (Dokladny et al. 2008) offers another mechanism by which the HSR preserves epithelial barrier integrity during heat stress. This increased epithelial barrier resistance may be a key factor in limiting gut associated endotoxin translocation during gut ischemia, heat stress, and low perfusion states such as the SIRS.

HSP ARE PRO-INFLAMMATORY

Intracellular HSP confer an anti-inflammatory state because they down regulate inflammatory cytokine production by immune effector cells, increase cell and tissue tolerance of cytokine mediated cytotoxicity, and attenuate epithelial barrier permeability changes. Paradoxically HSP, particularly Hsp70 and gp96, also orchestrate a vigorous and multifaceted inflammatory response when seen by immune effector cells in the local extracellular environment. The HSP are among the key molecules referred by Matzinger (2002) as “danger” signals for the immune system since they serve as co-stimulatory molecules for immune recognition. The release of Hsp70 into the extracellular environment is an area of current research interest, as there is evidence for active secretion as well as for their passive release from injured, necrotic, or virally infected cells. In this regard, a variety of cells, such as monocytes (Lancaster

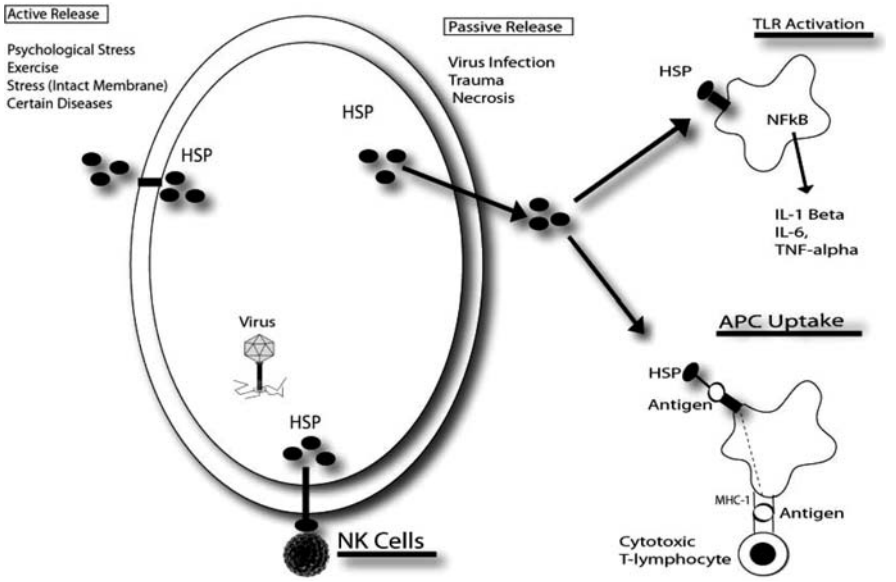

Figure 2. Mechanisms of HSP release into the extracellular environment

and Febbraio 2005), tumor (Broquet et al. 2003), glial (Guzhova et al. 2001), and B and T (Hunter-Lavin et al. 2004) cells actively release Hsp70 into the extracellular space. Additionally, extracellular Hsp70 (eHsp70) can interact with different peptides and microbial molecules to facilitate their detection to the immune system (Srivastava et al. 1994). Curiously, HSP have also been detected in the peripheral circulation of normal individuals with no signs of inflammatory or disease processes (Pockley et al. 1998). Thus the extracellular Hsp70 seems to be associated with both pathological and non-pathological conditions related to alerting the immune system to an altered cellular function. The model of pro-inflammatory HSP is described in Figure 2.

THE MECHANISM OF HSP RELEASE

Necrotic cell death, as consequence of mechanical injury or bacterial and viral infection, is the most evident mechanism to a cell release HSP. Experiments performed by Basu et al. (2000) demonstrated that necrosis induced by freeze and thaw result in the release of intracellular content, including Hsp70, into the extracellular environment. Interestingly, the authors also observed that during apoptotic stimulus induced by irradiation, the cell content seems to be packed into apoptotic bodies and the presence of eHsp70 is not detected even 24 h after death. Moreover, exposure of dendritic cells to necrotic but not apoptotic cell content elicited expression of maturation markers of

dendritic cells. Similar results were observed by Melcher et al. (1998) who observed that necrosis of tumor cells was associated with high immunogenicity and the presence of eHsp70. In contrast, induction of apoptosis showed low levels of eHsp70 and less immunogenicity. In humans, the presence of Hsp70 in the serum has been reported in patients after severe trauma or major surgical interventions such as coronary artery bypass (Pittet et al. 2002, Dybdahl et al. 2004). Dybdahl et al. (2005) also demonstrated that patients with acute myocardial infarction showed increased levels of serum Hsp70, and these values correlated significantly with important markers of cell necrosis (creatine kinase and cardiac troponin T). The presence of Hsp70 in the circulation would act as a danger signal or message alerting the antigen presenting cells to cellular damage of the surround or distant tissues and also activating the immune cells to a possible bacterial invasion.

Recent studies have also demonstrated active secretion of Hsp70 into the extracellular milieu by pathways other than cell damage. Under conditions of non-detectable cell death, eHsp70 has been shown to be released in glial cells (Guzhova et al. 2001), B cells (Clayton et al. 2005), and human peripheral blood mononuclear cells (Hunter-Lavin et al. 2004, Lancaster and Febbraio 2005). Although Hsp70 do not have a peptide leader sequence targeting secretion, it is suggested that the release occur via a non-classical pathway. In this regard, Bausero et al. (2005) demonstrated a possible mechanism by which HSP could be actively released from viable cells. The authors observed that Hsp70 could be released through exosomes when stimulated with certain inflammatory cytokines (IFN-y, IL-10). Additionally, Lancaster and Febbraio (2005) confirmed that exosome are the major pathway for secretory vesicular release of Hsp70. However, when the lipid rafts structure was disrupted through heat stress, the PBMCs were able to continue secreting Hsp70, suggesting additional pathways. Parallel with the active mechanism of release of Hsp70 in the extracellular milieu, secretory vesicles may fuse with the plasma membrane resulting in the expression of the Hsp70 in the surface of certain cells. In fact, different human tumors cells express Hsp70 in their plasma membrane (Multhoff et al. 1995, Melendez et al. 2006).

In whole organisms, serum Hsp70 has been detected in peripheral circulation of apparently healthy individuals (Pockley et al. 1998) and increased in response to different stressors, including certain diseases (Wright et al. 2000, Pockley et al. 2003), exercise (Walsh et al. 2001) and even psychological stress (Campisi et al. 2003). The source and mechanism of appearance of Hsp70 in the serum is not clear. It is possible that during disease states a combination of necrotic and active release by stressed tissue are responsible for Hsp70 in the serum. However, in conditions where cell necrosis or injury is not detectable, such as psychological stress and certain exercises, serum Hsp70 is possibly dependent on active release.

HOW DOES HSP70 INDUCE INFLAMMATION?

HSP are primarily considered a primitive intracellular molecule. The presence of HSP in the extracellular milieu of multicellular organism with immune surveillance

has been seen as an inducer of a highly immunogenic response. However, the immune effects of HSP released by necrotic are possibly different from the immune effects of HSP released during psychological stress. Additionally, different cells actively release HSP which can have different significance/interaction with immune effector cells. Here we will briefly discuss a direct LPS-like inflammatory response of extracellular HSP through Toll-like receptor (TLR) activation, as well as a role for HSP in antigen uptake of HSP-peptide complexes by antigen presenting cells and facilitated antigen presentation to cytotoxic T cells.

Cytokine Response Through TLR Activation

eHsp70 can bind to different cell surface receptors such as TLRs 2 and 4, resulting in signaling events and activation of antigen presenting cells. This activation results in a signaling cascade including stimulation of the inflammatory myeloid differentiation primary response, downstream IL-1 receptor associated kinase 4 (IRAK) and the NFκB signal transduction pathway. Asea (2007), for example, demonstrated that recombinant Hsp70 could signal through TLR-2 and TLR-4 with the involvement of CD14 of human monocytes stimulating rapid intracellular calcium efflux, activating NFκB and up-regulating pro-inflammatory cytokine secretion (IL-1β, IL-6 and TNF-α).

Enhanced Antigen Uptake and Presentation of HSP-Peptide Complexes

Extracellular HSP, particularly Hsp70, Hsp90, and gp96, serve as antigen carriers and facilitate antigen uptake by dendritic or antigen presenting cells (APC; Srivastava et al. 1994). Uptake appears to be through several mechanisms, including the alpha2-macroglobulin receptor (Binder et al. 2000). The HSP-peptide complex is more efficiently taken up by APCs than antigen alone. In addition to facilitated uptake, HSP appears to drive APC maturation and activate NFκB signal pathways (Basu et al. 2000). Finally, HSP facilitates antigen processing and transfer to a major histocompability complex I class molecule for presentation to cytotoxic T-lymphocytes.

NK Cell Activation

Hsp70 is expressed on the surface of tumors cells in culture (Multhoff et al. 1997) as well as in cells infected with several viruses, including HIV. The Hsp70 expression on tumour cells correlates with direct natural killer (NK) cell induced cytotoxicity and can be blocked by incubating target cells with antibodies directed against Hsp70 prior to NK cell exposure (Roigas et al. 1998).

UNDERSTANDING THE PARADOX OF THE PRO- AND ANTI-INFLAMMATORY ACTIONS OF HSP: THE HSP-VIRUS CONNECTION

Given the potency of intracellular HSP in blunting cytokine production and in increasing cell resistance to these same cytokines, it seems paradoxical, on first glance, that eHSP would have similar potency in activating the same cytokine pathways. It would seem even more paradoxical that these eHSP drive both NK cell killing and facilitate antigen uptake. We propose that this paradox represents an evolutionary adaptation of multicelled organisms to the threat of viral infection. It is beyond the scope of this chapter to detail the viral HSP connection, but this subject has been well reviewed by Sullivan and Pipas (2001). Most viral infections, whether bacteriophages, plant viruses, or animal viruses, are associated with an HSR. While this HSR was seen as a cellular response to stress or the appearance of new proteins within the cell, data from adenovirus studies reveal that, in fact, the HSP are essential for viral replication and so represent a viral rather than host cell adaptation (Glotzer et al. 2000). The HSP are employed by viruses as anti-apoptotic factors, as a means to move viral proteins to the cell nucleus, and in virion assembly. One consequence of the virus's requirement for HSP is that both free HSP as well as HSP-viral peptide complexes are released during virion release and/or virally-induced cell death. Since the HSP requirement and release by viruses occurs in both single and multicellular organisms, the immune systems of multicelled organisms evolved in a setting of HSP release. We propose that immune system evolution incorporated HSP and HSP-viral peptide release as a signal for activation. This sort of immune signaling is not unique. The immune/inflammatory activation of other primitive signals, such as CpG nucleotides, can also be considered under this paradigm.

DISEASE AND PATHOLOGIC STATES

Sepsis

Sepsis is a spectrum of diseases that encompasses infection, the SIRS, the development of organ dysfunction (severe sepsis), cardiovascular collapse (septic shock), and death (Hotchkiss and Karl 2003). Experimental and clinical data indicate that pro-inflammatory cytokines play a major role in sepsis-inducing systemic inflammatory response (Christman et al. 1995). To date previous studies have demonstrated that induction of the HSR protects animals in an experimentally induced sepsis model possibly by a reduction of the pro-inflammatory cytokines. As reviewed above, the transcription factor NFκB is an important regulator of inflammatory cytokines. Patients with sepsis show elevated levels of NFκB and it correlates with mortality rates (Bohrer et al. 1997). Interestingly, Schroeder et al. (1999) showed that peripheral blood mononuclear cells of patients with severe sepsis stimulated with LPS showed significantly less Hsp70 than healthy donors control subjects. Although these are two different studies, they indicate that the low Hsp70 observed

might be associated with a greater activation of the NFκB transcription factors, and consequently pro-inflammatory cytokines.

Heat Stroke-Associated Inflammation

Heat stroke is a life threatening condition where hyperthermia and its associated alterations in perfusion to critical tissues induce cellular dysfunction of critical tissues and the SIRS (Moseley 1997, Bouchama and Knochel 2002). While the mechanism by which heat activates this systemic inflammatory response is not completely clear, evidence is accumulating that hyperthermic stress drives inflammation through local tissue injury from ischemia/reperfusion as well as increased intestinal permeability, which facilitates endotoxin translocation across the intestinal lumen into the portal and splanchnic circulation. For example, it has been shown that levels of endotoxin in heat stroke patients are more than 1000-fold higher than normal healthy subjects (Bouchama et al. 1991). Additionally, patients suffering from heat stroke frequently present with pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6 and nitric oxide synthase, in the systemic circulation (Bouchama et al. 1991). The serum concentrations of IL-6, IL-1β, and INF-γ are inversely correlated with the severity of illness (Bouchama et al. 1993).

It has been demonstrated that elevations of core temperature result in early peripheral vasodilation with an associated fall in blood flow in the splanchnic circulation (Kregel et al. 1988). In this study, splanchnic dilation preceded terminal hypotension and death, implicating early splanchnic ischemia in the pathogenesis of heat stroke. Later studies using organ specific Hsp70 accumulation as a biomarker of stress demonstrate that the intestine and liver are the tissues most affected by hyperthermic stress (Flanagan et al. 1995, Beck et al. 1995). In vitro epithelial models also support early epithelial permeability as a key component of hyperthermic stress. These early changes occur at relatively mild temperatures (>38.3ºC) and are completely reversible (Moseley et al. 1994, Dokladny et al. 2006). Altogether, these data suggest that hyperthermia impairs the ability of cells to maintain critical cell-to-cell interaction probably affecting overall gut function and allowing LPS to leak into the portal circulation and eventually in the systemic circulation.

In addition to the inflammatory cascade unleashed by the leak of endotoxin as well as direct thermal and ischemic challenges to the gut, Hsp70 in the extracellular environment may also be involved in propagating the inflammatory response observed during heat stroke. Cells, especially from the comprising critical target tissues, undergoing the multiple stresses of hyperthermia activate the HSR, up-regulating the intracellular content of HSP (Flanagan et al. 1995). In cases of cell necrosis, HSP are released into the extracellular milieu, enhancing the immune/inflammatory response. Thus, there is a balance between intracellular HSP in inflammatory cells blunting inflammation and the release of HSP from injured cells activating inflammation. A model of extracellular Hsp70 related inflammation has been proposed for the maternal pre-eclampsia and the syndrome of hemolysis, elevated liver enzymes, and low

platelet count (HELLP, Molvarec et al. 2007, Madach et al. 2008). It is reported that patients with HELLP present an excessive maternal inflammatory response to pregnancy with cytokine-mediated endothelial damage in the liver, platelet activation and hepatocellular necrosis (Baxter and Weinstein 2004). In these diseases, extracellular Hsp70 might function like endotoxin, binding to specific receptors (CD14, CD91 and Toll-like receptors) on APC's, resulting in NFκB activation and the production of pro-inflammatory cytokines (TNF-, IL-1β and IL-6). Similarly in heat stroke patients, liver damage frequently is reported in heat stroke patients (Giercksky et al. 1999, Kew et al. 1970). Thus, extracellular Hsp70 might be important to drive to the systemic inflammatory response observed in heat stroke patients.

Aging Susceptibility to Heat Injury/SIRS

The aging process involves a progressive decline in physiological function, also accompanied by a reduction in stress tolerance. Several studies demonstrate that animals with advancing age have lower tolerance to a variety of physiological challenges, including heat stress (Hall et al. 2000, Zhang et al. 2003). In humans, an example of age-associated loss of stress tolerance is a higher occurrence of morbidity and mortality during periods of heat waves in older than younger individuals (Levine 1969, Semenza et al. 1996). Although the mechanism underlying the lack of thermotolerance in aging animals is not clear, previous studies have indicated that the HSR is impaired in older organisms (Liu et al. 1989, Fargnoli et al. 1990, Blake et al. 1991). The initial work done by Kregel et al. (1995) reported that the increases in liver and myocardial Hsp70 accumulation in response to non-exertional heat stress were attenuated in old rats. In a subsequent study, it was reported that the lower expression of Hsp70 was followed by extensive liver injury and higher mortality in older animals after two consecutives heat exposures (Hall et al. 2000). Interestingly, Hsp70 expression after the first heat stress was moderately reduced compared to the younger rats but by 48 h Hsp70 expression was dramatically lower. One interpretation of these results is that this lower Hsp70 response in the aged organism allowed tissue necrosis and release of Hsp70 in the extracellular milieu. The eHsp70, in turn, supported the inflammatory state which is similar to that reported in an aged organism (Dobbs et al. 1999). Thus, the HSR, through Hsp70 leak may be a driver of the greater susceptibility of systemic inflammatory response in aging.

Although this lower cellular Hsp70 accumulation seen in the severe heat stress model could be interpreted as an age- associated impairment of Hsp70 accumulation, curiously the aged organism is actually able to generate a similar Hsp70 response compared to the young organism when exercise is used as a stimulus (Kregel and Moseley 1996). Additionally, Nitta et al. (1994) observed reduced Hsp70-mRNA after 10 min of ischemia in aged rat hearts but, with longer period of ischemia, Hsp70 level was similar to young hearts. Thus, the data suggest that the aging process does not represent a loss of HSR but alterations in the quality or intensity needed for Hsp70 accumulation.

Inflammatory Bowel Diseases (IBD)

The inflammatory bowel diseases (IBD), which include the Crohn's disease and ulcerative colitis, are chronic relapsing idiopathic inflammatory disorders of the gastrointestinal tract. It is suggested that defects in both the barrier function of the intestinal epithelium and the local mucosal immune system promotes an inappropriate and ongoing inflammatory response to the presence of normal gut luminal flora (Podolsky 2002). Activation of immune cells by luminal antigens promotes the production of inflammatory mediators, including cytokines, chemokines and growth factors, resulting in tissue damage. Accumulated evidence suggests that TNF-α secreted by intestinal monocytes cells may play a role in the development of inflammatory bowel disease (Van Deventer 1997). It is reported that high levels of TNF-α are secreted by cells isolated from mucosal biopsy specimens of patients with IBD (Breese et al. 1994). In a cellular model of epithelial barrier, TNF-α caused an increase in intestinal tight junction permeability, shown by an increase in epithelial permeability (Ma et al. 2004). Furthermore, clinical and animal studies have shown that application of anti-TNF antibody decreased disease activity and caused transient remission confirming that TNF-α is an important mediator of IBD. The mechanism of the TNF-α induce an increase in intestinal epithelial tight junction permeability seems to be regulated in part by NFκB (Ma et al. 2004, Ye et al. 2006). Curiously, biopsies of patients suffering from Crohn's disease and Ulcerative Colitis show enhanced expression of Hsp70 (Ludwig et al. 1999). An interesting question is if the up regulation observed in patients with IBD is just a marker of cellular injury or if the cells are becoming more resistant to the inflammatory stress.

Additionally it has been hypothesized that Hsp70 may participate in the process of inflammatory bowel diseases when it is present in the surface of epithelial gut cells or exported to the local gut environment. As mentioned above, it is reported that biopsies of patients with inflammatory bowel disease show an enhanced epithelial expression of HSP (Ludwig et al. 1999). This enhanced epithelial Hsp70 expression may be involved in the surface presentation of altered self-protein to the immune system, activating immune cells or through molecular mimicry or homology to bacterial Hsp70 (Petrof et al. 2004). A more recent alternative hypothesis involving eHSP and bowel disease has been proposed by Fleshner et al. (2005). The authors suggest that eHSP released after exposure to stress binds to TLR4 expressed in epithelial cells, triggering endogenous bacterial translocation, increased phagocytosis, and an up-regulation of co-stimulatory molecules, resulting in an exaggerated inflammatory response. In this regard, Whittall et al. (2006) observed that both mucosal cells and circulating monocyte-derived dendritic cells from Crohns diseases patient showed an increased TNF-α production compared to control samples.

THE IMMUNO-PHYSIOLOGY OF ADAPTATION

As described above, the HSR modulate the immune/inflammatory response in both cultured cells and in animals. Numerous human and non-human studies demonstrate

that the physiologic stresses of exercise and whole body hyperthermia, as well as supplementation with agents such as GLN result in cellular Hsp70 accumulation. Early studies have shown that Hsp70 levels correlate with the environmental temperature of the ecological niche of a given species (Ulmasov et al. 1992). However, there remains considerable uncertainty whether the magnitude of the HSR in humans under these stresses is sufficient to alter tolerance to high core temperatures and inflammatory response.

Heat Acclimation and Adaptation

Heat adaptation can be divided into thermotolerance and acclimatization (Moseley 1997). The thermotolerant state is defined as the organism's ability to survive an otherwise lethal stress, including heat or endotoxin, from a prior heat exposure causing cellular accumulation of Hsp70. Some characteristics of the thermotolerant state are the ability of whole organism to tolerate a higher core temperature and endotoxin shock. The mechanism by which thermotolerant animals can tolerate higher core temperature may be due in part to the changes seen in cell culture studies, but there are also other important differences. Animal survival in thermotolerance depends more upon maintenance of tissue and organ function than on individual cell survival. When animals are subjected to severe heat stress, there is a marked fall in splanchnic perfusion (Kregel et al. 1988), which precedes terminal hypotension. This type of severe stress also results in the animals activation of a robust stress response in the gut and liver (Flanagan et al. 1995), suggesting an important role of gut and liver injury from thermal stress. The maintenance of intestinal epithelial tight junction barriers, for example, may block the passage of gut-associated endotoxin translocation. Thermotolerant animals may also maintain the liver Kupfer cells function during period of heat stress degrading endotoxin present in the portal circulation. Finally, thermotolerant animals may have a lower inflammatory response due to lower cytokine release from immune blood cells (Snyder et al. 1992, Ensor et al. 1994). Thus, it seems that cellular accumulation of Hsp70 is responsible for this thermotolerant state.

Heat acclimatized state is defined as an organism's ability to perform increased work in the heat because of improvements in heat dissipation brought on by repeated mild elevations in core temperature (Moseley 1997). Additionally, it is suspected that humans working in the heat, for example athletes, also demonstrate the ability to achieve higher core temperatures, in combination with the ability to sustain work at these higher core temperatures (Pugh 1967). This ability may not be the result of physiological changes observed in heat dissipation with heat acclimation but appears to result from cellular adaptations that allow continued function at higher temperatures, resembling the thermotolerant state. Studies using a heat acclimation protocol in humans induced the synthesis of Hsp70 in peripheral blood mononuclear cells (PBMC), suggesting a possible link between these forms of thermal adaptation (Yamada et al. 2007, McClung et al. 2008). More recently, we observed that heat

acclimation, which also resulted in cellular Hsp70 accumulation, had no effect on reducing PBMC injury after heat shock and did not alter anti- and pro-inflammatory cytokine release after LPS exposure (unpublished data). In contrast, *ex vivo* heat shock of 42.5ºC induced a much greater increase in PBMC Hsp72 compared to an acute bout of exercise in the heat on days 1 and 10 of heat acclimation. Thus, heat acclimation can induce changes in Hsp70 but the magnitude of induction may not able to induce a state of thermotolerance or modify the cytokine response to endotoxin.

CONCLUSION

The heat shock response has clear and potent effects on inflammation. While it is tempting to construct simple linear models of cause and effect, the reality is one of complex interactions, paradoxes, and balances. Using the highly stress-inducible Hsp70 as an example, we have seen that it can modulate inflammation by interrupting NFκB signaling as well as creating cytokine resistance in target cells. Hsp70 also directly enhances the functional resistance of multicellular tissues, such as epithelial barriers, to different stresses. This enhanced epithelial barrier resistance, in turn, may decrease endotoxin translocation into the circulation and so may indirectly alter cytokine production by decreasing the stimulus for cytokine production. Further, while the role of HSF-1 has been seen as limited to HSP transcription, more recent data suggests that it, too, directly modulates cytokine production. The recent observations that HSF-1 directly alters transcription of the tight junction protein, occludin 1, also suggest a much broader role for the HSR transcription machinery.

At the same time, paradoxes abound. Hsp70, which has been demonstrated to inhibit endotoxin induced IL-6, and has been correllated to GLN associated decreases in IL-6 in critically ill patients and animal studies is temporally linked to exercise induced IL-6 production in exercise. In addition, GLN exposure in exercise enhances muscle IL-6 production, suggesting a role for the HSR in this increase. Secondly, we have briefly reviewed the potent pro-inflammatory effects of the HSP, again seen through the Hsp70 model. This response is quite broad based. In that it involves inflammatory cytokine production, NK cell targeting, and marked enhancement of antigen presentation resulting in a specific cytotoxic T cell response. We have speculated that this pro-inflammatory response is an evolutionary adaptation to the role of HSP in the replication of viruses. This requirement extends across viruses infecting eukaryotic and prokaryotic life forms, demonstrating its ubiquity and high degree of conservation. Finally, studies using acute exercise or acclimation protocols in humans, and which have demonstrated Hsp70 alterations have largely failed to alter cytokine levels from stimulated cells. This failure may reflect an insufficient level of Hsp70 induction compared to the robust inflammatory stimulation. Clearly, there are a number of disease states where the ability to activate Hsp70 in cells, limit eHsp70, or create a specifically targeted eHsp70 response offers the opportunity to address a wide variety of disease states.

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CHAPTER 5

HSP REACTIVE T CELLS ARE ANTI-INFLAMMATORY AND DISEASE SUPPRESSIVE IN ARTHRITIC DISEASES

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Abstract: Immune responses to certain heat-shock proteins (HSP) develop in virtually all inflammatory diseases; however, the significance of such responses is only now becoming clear. In models of experimental arthritis, HSPs can prevent or arrest inflammatory damage, and in initial clinical trials in patients with chronic inflammatory diseases, including rheumatoid arthritis, HSP peptides have been shown to promote the production of anti-inflammatory cytokines, indicating immunoregulatory potential of HSP. Heat shock proteins, also called stress-proteins, are ubiquitous self-antigens that are over-expressed in inflamed tissues. For some reason, the prokaryotic homologous proteins, present in every bacterial species, are dominantly immunogenic. This is striking, especially given the fact that these proteins have large areas of sequence homologies with the host (mammalian) counterparts. Furthermore, in experimental models of arthritis, immunisation with bacterial heat shock proteins has been seen to lead to inhibition of disease development. In addition oral or nasal administration has similarly been seen to lead to disease inhibition. Based on the experimental evidence collected, it becomes attractive to suppose that the exposure to homologues of these self antigens, as present in for instance the bacterial intestinal flora, has a decisive impact on the regulation of self tolerance at the level of T cells. If so, it becomes attractive to use such proteins or their derivative peptides for modulation of inflammation relevant T cells as an antigen specific immunotherapy approach, without the immediate necessity of defining disease specific auto-antigens

Keywords: Heat shock proteins; arthritis; T cells; immune regulation

Abbreviations: APC, antigen presenting cells; APL, altered peptide ligands; BCG, Bacillus Calmette-Guérin; DC, dendritic cells; DMARDs, disease-modifying anti-rheumatic drugs; EAE, experimental autoimmune encephalomyelitis; HSP, heat shock proteins; IBD,

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inflammatory bowel disease; IL-10, interleukin-10; MHC, major histocompatibility complex; RA, Rheumatoid arthritis; TCR, T cell receptor; Tg, transgenic; TGF-β, transforming growth factor-β; TLR, toll like receptor; TNFα, tumor necrosis factor-alpha;

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common human autoimmune diseases characterized by chronic inflammation of the synovium of diarthrodial joints (Lee and Weinblatt, 2001). It can lead to long-term joint damage, resulting in chronic pain, loss of function and disability. Primarily the small joints of the extremities are affected, but as the disease progresses more of the large joints become involved too. The chronic inflammatory process induces changes in the cellular composition (cellular infiltration) and the gene expression profile of the synovial membrane, resulting in hyperplasia of the synovial membrane, which causes structural damage of cartilage, bone and ligaments (Klippel, 2001). Extra-articular disease affecting a variety of organs occurs in the majority of patients and is a significant factor in morbidity and mortality of people with RA (Pincus and Callahan, 1989). The severity of RA encompasses a wide spectrum, ranging from self-limiting disease to chronic progressive disease, causing varying degrees of joint destruction and clinically evident extra-articular organ involvement.

RA occurs in 0.5–1.0% of the population worldwide (Alamanos and Drosos, 2005; Silman and Pearson, 2002). The prevalence is about two to three times more common in women than in men. Although the cause or causes of RA remain elusive, the general consensus is that factors contributing to its occurrence and course (clinical heterogeneity) are probably both genetic and environmental. The main risk factors for the disease include genetic susceptibility, sex and age, smoking and infectious agents. In addition, hormonal, dietary, socioeconomic, and ethnic factors seem to contribute (Alamanos and Drosos, 2005; Silman and Pearson, 2002; van der Helm-van Mil et al., 2005). The major goals of treatment of arthritis are to reduce pain and discomfort, prevent deformities and loss of joint function, and to maintain a productive and active life. Inflammation must be suppressed and mechanical and structural abnormalities corrected or compensated by assistive devices. The introduction of new therapies such as tumor necrosis factor-alpha (TNFα)-blocking agents and new treatment strategies, especially early and aggressive therapy, including combinations of several disease-modifying anti-rheumatic drugs (DMARD) have improved the outcome for RA patients (Voll and Kalden, 2005). Unfortunately, these therapies form not a cure, as continuous systemic immunosuppression is required to maintain clinical benefits. Consequently, the long-term side effects are unsure while the costs are high (Fleischmann and Yocum, 2004; Goronzy and Weyand, 2005).

EXPERIMENTAL MODELS FOR RA

To understand the complexity of the pathogenesis of RA and for preclinical testing of new therapeutic agents, animal models are a necessity. To be able to evaluate

and select suitable animal models for RA it is crucial to reproduce some of the basic features of RA in such models (Holmdahl, 2000). Hallmarks are first, tissue-specificity; RA is characterized by a tissue-specific, inflammatory attack affecting diarthrodial joints. Although systemic manifestation can be prominent, the predominant inflammatory attack is directed towards peripheral joints. Second, chronicity; in RA chronicity is an essential characteristic. The disease course may proceed with identifiable relapses, but there is usually steady progression of joint destruction. Third, MHC class II association; the genetic influence is significant though not prominent and points towards an important role of class II genes in the MHC. In particular, certain structures near the peptide-binding pocket of HLA-DR4 molecules are highly associated with RA (Nepom, 2001; Roudier, 2000).

Many models have been described and each of them represents different aspects of the disease. The models described for RA so far can be divided into three principal groups: (1) cartilage protein-induced, (2) adjuvant induced, and (3) spontaneous. As arthritis is mediated by a specific immune attack on cartilage in peripheral joints, it is not surprising that several cartilage proteins, such as PG (Glant et al., 2003), CII (Brand et al., 2003), gp39 (Verheijden et al., 1997) and cartilage oligomeric matrix protein (COMP) (Carlsen et al., 1998) have been shown to be arthritogenic in different animal strains.

In our recent studies we used the proteoglycan (aggrecan)-induced arthritis (PGIA) model because this model reproduces several features of RA in which we are especially interested. PGIA is a chronic arthritis model which makes it especially useful to test immunomodulating agents over a longer period. Furthermore, PGIA is an antigen-induced arthritis model fundamentally controlled by T cells. This made it possible to generate a T cell receptor (TCR) transgenic (Tg) mouse (Berlo et al., 2005) and study the role of antigen-specific T cell responses (Berlo et al., 2006). In addition, PGIA is induced by a cartilage matrix component and there is growing evidence that, at least in a subset of patients with RA, antigen-specific T cell responses to cartilage matrix proteins do develop (Boots et al., 1997; Glant et al., 1980; Goodstone et al., 1996; Guerassimov et al., 1998; Klareskog and McDevitt, 1999; Li et al., 2000; Zou et al., 2003). Among the candidate autoantigens (Glant et al., 1992; Poole et al., 1988), cartilage PG aggrecan is one of the target autoantigens in RA joints (Boots et al., 1997; Glant et al., 1980; Goodstone et al., 1996; Guerassimov et al., 1998; Klareskog and McDevitt, 1999; Li et al., 2000; Zou et al., 2003).

Aggrecan (Figure 1) is a complex macromolecule consisting of a large core protein to which glycosaminoglycan and oligosaccharide side chains are covalently attached (Glant et al., 1998a; Rosenberg and Buckwalter, 1986). The core protein of aggrecan is heavily degraded during inflammatory processes, which results in the loss of function of articular cartilage (Glant et al., 1992; Poole et al., 1988, 1994).

Immunization of BALB/c mice with partially deglycosylated human PG (hPG) induces chronic progressive polyarthritis and spondylitis (Glant et al., 1987). This PGIA model has many similarities with human RA, as indicated by clinical assessments, radiographic analyses, scintigraphic bone scans, laboratory tests, and

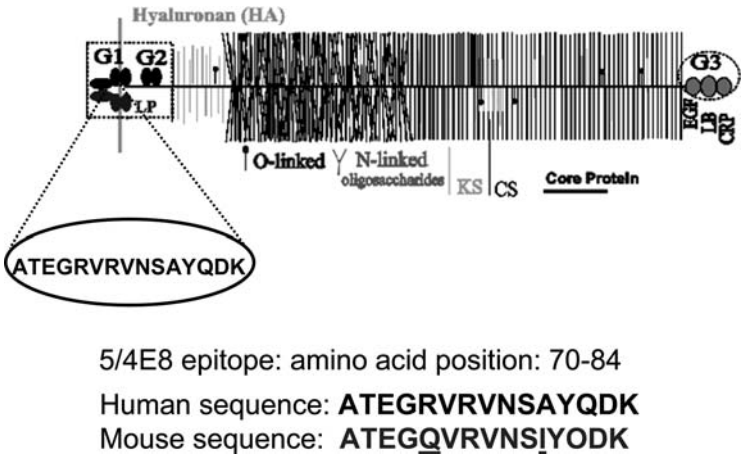

Figure 1. The molecular structure of human proteoglycan. The major T cell epitope of the link protein (LP) is indicated. Transgenic mice with a T cell receptor specific for this epitope are highly arthritis susceptible. The amino-acid sequence of this epitope is conserved and closely related to the mouse epitope

histopathology of peripheral joints (Glant et al., 1998b, 2003, 1987; Mikecz et al., 1990). The development of the disease is based on T and B cell responses cross-reactive between the immunizing human and mouse (self) cartilage PG (Glant et al., 1998b, 2003; Mikecz et al., 1987). Although the production of mouse PG-specific antibodies precedes inflammation and shows a high correlation with the incidence of arthritis, neither these antibodies nor PG-specific B cells alone can transfer the disease to naïve syngeneic mice (Mikecz and Glant, 1994, 1996; Mikecz et al., 1990). Adoptive transfer of PGIA requires the presence of both T and B cells from arthritic animals (Bardos et al., 2002a, b; Mikecz et al., 1990), and a rapid accumulation of mouse PG-specific Th1 cells in the synovium appears to be the most critical component of the development of arthritis (Glant et al., 2003; Hollo et al., 2000). Once an animal develops arthritis, more and more joints become involved and repeated "spontaneous" episodes of inflammation result in complete deterioration of articular cartilage and lead to deformities of the peripheral joints (Glant et al., 2003).

THE ESSENTIAL CONTRIBUTION OF ANIMAL MODELS IN ANTIGEN SPECIFIC IMMUNOTHERAPIES

Animal models have been pivotal in the development of antigen specific immunotherapies. Studies in antigen induced disease models have demonstrated that the inducing antigen itself or related compounds such as altered peptide ligands (APL) (Miller et al., 2007; Wauben et al., 1992) can be used for the down-modulation of disease producing immune effector T and B cells, even in established disease. In

addition, a variety of effector cells and mechanisms associated with such modulation, such as regulatory T cells and modulatory cytokines TGF-β and IL-10, have been uncovered in animal models. Despite the fact that in some of the animal models auto-antigens are used that have also been suggested to play a role in human autoimmune diseases, the development of specific therapeutics for use in humans that are directly based on the results of these animal studies has not been possible or very difficult, partially given the significant differences in antigen selecting MHC between rodent species and humans. Also, in most human autoimmune diseases the critical antigens involved are still to be identified and are possibly variable between different individuals.

In arthritis both antigen induced models (e.g. proteoglycan and collagen induced arthritis) and so-called adjuvant induced models are available. The latter models are induced with mycobacteria in oil or just synthetic or natural oily adjuvants. Besides the fact that the adjuvant models may resemble human disease – mycobacteria are known to produce arthritis also in humans such as seen as a side effect of BCG immunotherapy in for instance bladder cancer (Ochsenkuhn et al., 1990) – they have the advantage of not to depend on specific antigens (Van Eden and Waksman, 2003).

The immunomodulatory activities of mycobacterial HSP60 were discovered in the rat mycobacteria (adjuvant) induced model (van Eden et al., 1988) and subsequent testing in other both antigen and adjuvant induced models revealed the broad immunomodulatory qualities of this molecule. Although the mechanisms involved are likely to be manifold given the complex biology of HSP, there is accumulating evidence that conserved sequences of mycobacterial HSP60 are inducing regulatory T cells with the capacity to cross-recognize self-HSP60 over-expressed in stressed inflamed tissues. Such a "disease specific auto-antigen" independent bystander regulatory activity would explain why HSP are disease suppressive not only in adjuvant arthritis but also in other inflammatory conditions such as antigen induced arthritis, diabetes, atherosclerosis and several others (van Eden et al., 2005).

The efficacy of the targeting of regulatory T cells to antigens specifically expressed at sites of inflammation was recently demonstrated by Guichelaar et al. showing that proteoglycan specific T cells transduced with the IL10 gene suppressed proteoglycan induce arthritis in mice very effectively. Similarly transduced T cells with other T cell specificities were shown not to have such an effect (Guichelaar et al., 2008a).

SELF HSP CROSS-REACTIVE T CELLS HAVE ANTI-INFLAMMATORY REGULATORY ACTIVITY

Following the original definition of HSP60 as a critical T cell antigen in the rat adjuvant arthritis models (van Eden et al., 1988), the arthritis suppressive protective nature of the T cell responses induced by HSP60 was seen in various models of induced autoimmune inflammation, such as diabetes type I, atherosclerosis and

EAE (reviewed in (van Eden et al., 2005)). Epitope mapping of the arthritis protective mycobacterial HSP60 molecule in Lewis rats by Anderton et al. (1995), revealed the presence of one conserved epitope, showing very high sequence homology with its mammalian counter sequence present in rat (and human) HSP60. The T cells responding to this conserved 256–265 sequence, cross-responded to the mammalian homologue peptide and were seen to induce protection against arthritis in T cell transfer experiments. This was not seen for eight additional, less conserved mycobacterial T cell epitopes. In other words, only self-HSP cross-responsive T cells were seen to transfer protection in this experimental arthritis model. And this was found not only in the mycobacteria induced adjuvant arthritis, but similarly in the model where arthritis is induced by a fully synthetic (non-microbial) oily adjuvant, called avridine.

Subsequent experiments have shown the same principle for mycobacterial HSP70 in the rat model of adjuvant arthritis (Wendling et al., 2000). More recently, also in Balb/c mice mycobacterial HSP70 epitopes were mapped and again peptides based on conserved epitopes were found that had protective effects in the mouse PG arthritis model (Wieten et al., in preparation).

WHY ARE SELF-HSP RESPONSIVE T CELLS PROTECTIVE IN ARTHRITIS?

In the rat adjuvant arthritis model, various antigens were tested for their capacity to induce regulatory T cell responses. A selection was made of immunogenic proteins, present both in prokaryotes and eukaryotes and of a relatively conserved nature, given their (usually) enzymatic functions in nature. In this analysis in turned out that HSP70 induced IL10 producing T cells and also an antibody profile compatible with a Th2 response. In contrast to this, control antigens (G3PDH, aldolase, SOD etc.) induced a pro-inflammatory cytokine profile in T cells: IFNγ, TNFα.

There are a number of possible mechanisms responsible for the immunoregulatory phenotype of HSP specific T cell responses. A first major possible mechanism is the imprinting of a tolerant phenotype at the level of the gut mucosa due to contact with intestinal microflora. Mechanisms of mucosal tolerance may produce a regulatory phenotype especially in T cells that see HSP epitopes in multiple bacteria of the gut microbiota. Self-evidently, due to repeated and almost continuous encounter, these are T cells that see conserved epitopes in the first place.

A second possible mechanism contributing to this regulatory phenotype is the recognition of self-HSP epitopes in stressed tissues (and cells) which are not representing professional APC. As every cell will produce HSP under stress, MHC presentation of HSP epitopes in the absence of co-stimulatory molecules will promote tolerance in the form of anergy or IL-10 production. In addition the recognition of closely related but not identical self HSP by microbial HSP specific T cells may induce a partially agonistic response, similar to what is seen after stimulation with so-called altered peptide ligands or APL.

Thirdly, cell stress seems to lead to production of IL10 in many different cells types (Stordeur and Goldman, 1998). In other words, stressed cells create a regulatory T cell promoting environment in their vicinity, most likely a natural response to foster dampening of the consequences of local stressful circumstances.

TREG AND TARGETING REGULATION TO SELF ANTIGEN IN AUTOIMMUNE DISEASE

Autoimmune diseases feature excessive pro-inflammatory T cell responses to self-antigens. In particular effector Th1 cells and Th17 cells, through producing interferon-γ and interleukin-17 (IL-17) respectively, are held responsible for the induction or perpetuation of local inflammation in tissues (Bettelli et al., 2007; Liew, 2002). Several subsets of T cells that can downregulate excessive pro-inflammatory autoimmune responses have been described. Studies on mechanistic functions of such Treg cells (predominantly Tr1 cells, Th3 cells and $Foxp3^{+}CD4^{+}CD25^{+}$ natural Treg cells) have led to a current hypothesis that autoimmune disorders are the result of a disturbed immune balance of autoantigen-specific Treg versus effector T cells (Bluestone, 2005; von Herrath and Harrison, 2003). For example, studies in arthritis patients have shown that inflammation and increased numbers of Th1 cells correlate with reduced numbers of regulatory T cells producing the immunomodulatory cytokine interleukin-10 (Yudoh et al., 2000).

Although immunogenic self-antigens vary among different individuals due to variety of HLA-type, T cell receptor repertoire or antigen processing, many different self-antigens have been identified to which T cells from arthritis patients respond. T cells restrict their responses to the micro-environment where these self-antigens are available for presentation to antigen-specific T cells. Therefore, exploitation of immunosuppressive Treg cells that can recognize self-antigens in inflamed tissues is a promising tool for long-term and disease-specific therapeutic intervention in inflammatory autoimmune responses.

Several approaches have been employed to boost the anti-inflammatory capacities of self-antigen responsive Treg cells (Bluestone, 2005; von Herrath and Harrison, 2003). In vitro screening of patient T cells from peripheral blood or inflammatory lesions has generated candidate-epitopes from self antigens. Application of such antigens via appropriate routes, like oral or nasal administration, has been explored successfully to stimulate beneficial regulatory antigen-specific responses to cartilage-derived antigens in animal models of arthritis and tissue-antigens in other autoimmune disorders (Broere et al., 2008; von Herrath and Harrison, 2003). Despite these promising results however, first clinical trials testing oral administration of antigen have not shown significant clinical benefit. This indicates that further research on antigen-dose, bioavailability of antigens and Treg biology is needed for development of appropriate antigen-specific immunotolerogenic regimes (von Herrath and Harrison, 2003).

In addition to in vivo induction and expansion of Treg cells, our current knowledge on T cell differentiation has also paved ways to new approaches for *ex vivo*

generation of Treg cells that target the inflammatory response against self-antigens (Bluestone, 2005). T cells with regulatory properties can be generated from conventional $CD4^+CD25^-$ in vitro, for instance, by TCR-triggering in the presence of TGF-β (Chen et al., 2003). Moreover, existing Treg cells can be expanded in vitro by stimulation with anti-CD3 antibodies and co-stimulation with anti-CD28 antibodies in the presence of high doses of IL-2 (Bluestone, 2005). Therefore, Treg cells that are selected against appropriate self-antigens and expanded in vitro may be syngeneically transferred into the patient. A drawback of such a therapy, however, may be that arthritis patients have relatively small populations of functional Treg cells directed to the self-antigens that are involved in disease. In addition, Treg cells often show rather low proliferation in response to antigenic stimulation in vitro, compared to conventional T cells. Therefore, using in vitro screening for candidate antigen-specific Treg cells, it may be difficult to pick up these small numbers of readily existing Treg cells. Also the lack of a unique Treg marker to be used for selection of true and uniform populations of Treg cells is still thwarting application of defined Treg cells. Therefore, definition of unique Treg markers and extending insights in differentiation and proliferation of Treg cells will contribute to safe and efficient clinical use of Treg cells.

Another method to generate T cells with regulatory properties has evolved from recombinant-DNA technology. Cloning of genes with proven immunosuppressive properties and protocols for gene transfer to cells allows provision of T cells with active recombinant genes encoding immunoregulatory or anti-inflammatory molecules.

The recent knowledge and explosion of interest in Treg biology and hallmark molecules of Treg cells, such as Foxp3, inducing a natural $CD4^+CD25^+$ Treg phenotype upon retroviral transduction of murine (Hori et al., 2003) and human T cells (Aarts-Riemens et al., 2008), and IL-10, produced by Tr1 cells (Groux et al., 1997), has brought the field of so called adoptive cellular gene transfer closer to therapeutic "mimicking" of Treg cells. Gene transfer of the Treg marker Foxp3 to autoantigen-specific T cells has not been shown in arthritis models yet, though Foxp3-transduced T cells have been shown to suppress disease in other models of inflammation. Another molecule that has a central role in immunosuppression by Treg cells is the cytokine IL-10. This cytokine is not only produced by subsets of Treg cells, but also by B cells and antigen-presenting cells. IL-10 can suppress antigen-presentation to T cells, production of pro-inflammatory cytokines and, therefore, also activation of effector T cells. For these reasons the IL-10 gene can be a good candidate for therapeutic intervention in inflammatory disorders. Recently, we have shown that gene transfer of IL-10 to arthritogenic T cells induce Treg cells that suppress arthritis induced by artificial antigens (Setoguchi et al., 2000) and chronic arthritis induced by the cartilage-antigen proteoglycan in mice (Guichelaar et al., 2008a). IL-10-transduced Treg cells, resembling Tr1 cells (Guichelaar et al., 2008b), target chronic arthritis in an antigen specific way since only T cells carrying a TCR for a cartilage-antigen (proteoglycan) suppressed disease. Moreover, in the chronic proteoglycan-induced arthritis model in mice,

IL-10 transduced proteoglycan-specific T cells were more effective in suppressing inflammation than T cells that were transduced with genes encoding IL-1RA, TNFα-R or IL-4 (Guichelaar et al., 2008b), indicating strong potency and a central role of IL-10. Intriguingly, IL-10-transduced T cells promoted the endogenous expression of immunoregulatory IL-10 in non-transferred host cells of the treated animals. This suggests that antigen-specific IL-10-transduced T cells do not only antigen-specifically target pro-inflammatory effector cytokines, but also propagate the regulatory immune response to restore the disturbed immune balance that is responsible for the inflammation. Be this as it may, the selective arthritis suppressive potential of cartilage PG specific T cells in the experimental model, has shown that T cells can be targeted to sites of inflammation, on the basis of their specificity for antigen expressed at sites of inflammation. Therefore HSP are attractive targets for antigen specific therapies which involve the suppression inflammation through regulatory T cells.

THE IMMUNOMODULATORY EFFECTS OF HSP ON APC

It is clear that regulatory T cells play an important role in immune homeostasis. And HSP seem to fulfill a very specific immunoregulatory capacity, through the regulatory role of self HSP-specific T cells. More recently other immunologically relevant functions of HSP have been discovered and HSP have been suggested to directly activate the innate arm of the immune system via activation or modulation of antigen presenting cells. HSP can directly activate the immune system through surface receptors such as toll like receptor (TLR)2, TLR4, CD91, CD40 and CD14. (Pockley, 2003; Quintana et al., 2004). More recently CCR5 was added to the list of potential receptors (Floto et al., 2006; Whittall et al., 2006). Via these receptors HSP can activate dendritic cells and enhance both MHC I and MHCII mediated antigen presentation. However, since the activating effects of HSP in in vitro culture systems is similar to the effects of low dose LPS and lipoproteins this data raised the concern that observed effects were due to bacterial contaminants of the recombinant HSP preparations (Gao and Tsan, 2003, 2004; Wang et al., 2005). Eventhough this can not be entirely excluded, several reports show that also highly purified HSP can modulate DC's and macrophages. For example HSP derived from murine liver and kidney was able to activate DCs and macrophages (Panjwani et al., 2002). In addition in vivo application of "clean" HSP70 did not induce DC maturation in splenocytes whereas "dirty" HSP70 or LPS controls did. Moreover, data from our own laboratory confirm the observation that a "clean" HSP protein batch only mildly influences DC maturation, based on the expression of costimulatory molecules. However, DC's that were incubated with HSP70 produced significantly higher levels of IL10, indicating a more regulatory phenotype of the DC. This data suggests that HSP might induce a microenvironment that is favorable for the induction of regulation.

Apart from these technical difficulties, it is obvious from literature that HSP have an important function in immune regulation not only via the activation of specific

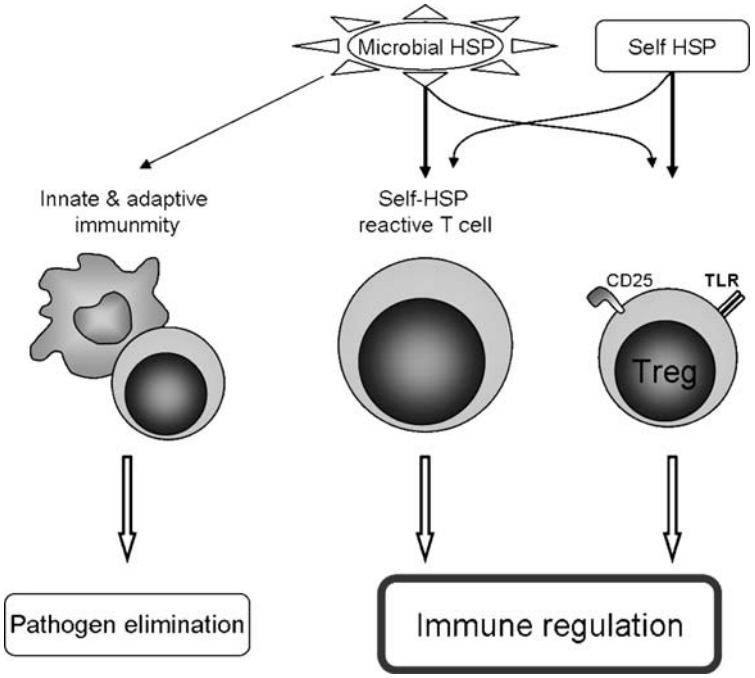

Figure 2. The complexity of HSP immune system interactions. Microbial HSP trigger both innate and adaptive immune reactions leading to pathogen elimination in infection. Self-HSP are involved in immune reactions leading to immuneregulation. Conserved epitopes of microbial HSP trigger self HSP-reactive T cells, which can induce immunoregulation. In the kinetics of microbial HSP immunity, pro-inflammatory responses are followed by inflammation dampening immune regulation

T cells but also via direct effects in the innate immune system. Thereby one can imagine that incubating DCs with HSP not only induces HSP peptide presentation to T cells, but that this also modifies the context of immune activation (Figure 2). As said, APC cultured in presence of HSP70 produce more IL10 (Detanico et al., 2004; Motta et al., 2007). In addition HSP70 treatment reduced T cell proliferation to a mitogenic stimulus (Motta et al., 2007). Summarizing all this data, HSP can both induce DC with a regulatory phenotype, and produce HSP specific regulatory T cells. The combination of these two features makes HSP attractive for the antigen specific manipulation of autoimmune diseases.

As described above, especially self HSP specific cross-reactive T cells show regulatory potential. It is obvious herein that the presenting DC phenotype and the origin of HSP can differentially affect the subsequently induced immune response. HSP are intracellular proteins, however their effect on the immune system has mainly been studied by adding exogenous HSP either from bacterial origin or recombinant self. Release of HSP in the extracellular matrix only occurs during necrotic cell death and not during apoptosis. It is feasible that under conditions of necrosis different

immunological intervention is needed, compared to others forms of immunological stress that will result in upregulation of HSP without cell death. Overexpression of HSP70 in macrophages inhibited subsequently LPS induced cytokine release and T cell stimulatory capacity (Ding et al., 2001). We ourselves have seen that up-regulation of HSP70 in DC via heat shock induced HSP up-regulation, also induced DC with a regulatory phenotype with higher IL10 production and a reduction in the subsequently induced T cell response. This was not due to reduced antigen presenting capacity of these DC's (unpublished observations).

Based on this data the protective potential of bacterial HSP immunizations in animal models of autoimmune disease will be the net effect of inducing DC with a regulatory phenotype and the induction or expansion of self-HSP specific regulatory T cells.

Since only cross-reactive peptides seem to induce a regulatory T cell response involving the induction of IL-10, self-HSP reactive responses might be regulatory while bacterial HSP epitopes which are uniquely present in microbial HSP might induce proinflammatory responses. However, which factors contribute to the selection and dominance of such self-reactive epitopes upon bacterial HSP immunizations is unclear. It is obvious that such knowledge might help the further development of the therapeutic potential of HSP.

BRIDGING THE TRANSLATIONAL GAP BETWEEN MODEL AND DISEASE IN ANTIGEN SPECIFIC IMMUNE INTERVENTIONS

Until now the success of translating effective antigen specific regulation based interventions into human therapies has remained limited. This is for several obvious reasons. Regulatory agencies (and in many cases commercial investors) expect biological therapies to develop along the lines of classical drug therapies: one optimal dose to be effective in most of the patients with cure of disease as the necessary endpoint. This however is difficult to achieve. Animal models can show optimal dosages relative easily, as the recipients are usually inbred and homozygous for critical genetic elements such as MHC. In patients dosing will depend on complex genetics and more variable environmental factors. In addition the disease in most cases will be less homogeneous; patients will be in different activity stages of their disease and will have different earlier immune experiences. Cure of disease will depend on the immunological effect that can be reached with the immune intervention. Therefore the critical endpoints need to be based on immunological monitoring. Such monitoring will reveal responders and non-responders. For this monitoring, various assays are now available and are still under development. As a consequence of this variable responsiveness, the more attractive antigenic compounds for therapies will be those that have shown their activities in multiple (inbred) strains of animals and preferably in multiple disease models. HSP are examples of compounds that have shown broad activities in various disease models (van Eden et al., 2005).

Human trials were in the majority of cases carried out in end-stage diseases. From what we understand now of the immunology of the progressed and chronic stages of inflammatory disease, one may predict that antigen specific regulation might not be effective by itself in these stages of disease. In these cases combinations of antigen specific therapies with more generalised immune suppressive regimens may be needed (Prakken et al., 2007).

Rapid progress is now being made in the elucidation of the cellular mechanisms of antigen specific interventions. With the recent identification of specialised regulatory T cell subsets (Sakaguchi et al., 1995), it now will become possible to further guide antigen recognition in the direction of induction of immune modulation. $Foxp3^+$ $CD25^+$ regulatory T cells have been shown already to be triggered by orally administered HSP60 peptides in a mouse model for atherosclerosis (van Puijvelde et al., 2007). In addition oral, joint cartilage derived, proteoglycan has been shown to trigger regulatory T cells which were seen to directly interfere with joint PG specific inflammatory effector T cells in vivo (Broere et al., 2008). To guide mucosal antigen recognition to immune modulation by careful antigen (epitope) selection or by other additional means will be critical, given the observations that oral and intranasal autoantigen administration during clinical disease may also exacerbate disease, as was shown previously in various animal models (Hanninen and Harrison, 2004).

GOOD CHANCES FOR HSP DIRECTED IMMUNE MODULATION AS A NOVEL ADDITION TO CLINICAL ARTHRITIS SUPPRESSIVE THERAPIES

Immune manipulation through HSP proteins or peptides are examples of antigen or epitope specific immunotherapies. The effects of such interventions in chronic and established diseases with lasting and progressive immune aberrations, such as seen in advanced RA, are expected to be moderate.

As with most other immunological tolerance enhancing strategies, chances to correct a dysfunctional immune status with antigens are best at very early stages of disease. Therefore, with the advent of novel means of genetic susceptibility testing and early diagnostics there will be an increasing need for effective but also subtle therapeutic interventions with no or minor side effects. Antigen specific immunotherapies based on HSP may therefore offer attractive possibilities.

In addition, antigen specific immunotherapies may become attractive in conjunction with the recently developed biologics. TNF inhibitors have shown great efficacy in halting the inflammatory process in RA. And further biologicals acting in a similar manner are now developed at a rapid pace. However, there are several aspects of these new biologicals that seem to seriously impair their rate of success. First of all, there are side effects through their generalized capacity to hamper immune defense, leading to lymphomas and, much more seriously, life threatening infections. Secondly, the new biologicals do not induce permanent cure. Disease will relapse as

soon as therapy is interrupted. Thirdly, most of the novel biologics are very expensive. Antigen specific immunotherapies are therefore attractive alternatives that may well find their niches in being used as so-called post-biologics. Upon cessation of biologic administration, antigen specific immunotherapy may be installed to prolong the anti-inflammatory effects of the biological and possibly to lead to permanent cure. Alternatively, the antigen specific intervention may be administered in conjunction with the biologic, creating a possible synergistic effect, which would make it possible to administer the biological at a lower dose. The latter has been shown to be possible in experimental arthritis combining TNF inhibition (Enbrel) with HSP peptides (Roord et al., 2006).

As an aside, microbial HSP interactions with T cells (and innate receptors) also might constitute a molecular basis for the hygiene hypothesis, which links exposure to environmental microbes to a lower prevalence of immune-mediated disorders. If so, the increasing immune dysfunction, leading to a higher prevalence of immune-mediated diseases, such as allergies and autoimmune chronic diseases (diabetes, MS, IBDs), might possibly be countered by exposure to microbial HSP. The prevention of such diseases in experimental models by administering HSP proteins and peptides is compatible with such a possibility and seems to suggest that HSP could act as vaccines with a capacity to restore immune fitness.

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CHAPTER 6

HEAT SHOCK PROTEINS IN VASCULAR DISEASE

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Abstract: There is growing evidence that heat shock proteins (HSP), a family of stress-inducible proteins may be involved in the pathogenesis of atherosclerotic vascular diseases. Here we systematically review the evidence behind this notion. A detailed literature search and extensive bibliographic review of literature relating to HSP and atherosclerotic vascular disease. Atherosclerotic vascular disease is classified into four main areas of presentation: carotid, coronary, aortic and peripheral vascular disease, for consideration in this review. In each of these vascular diseases, the evidence linking HSP and atherosclerosis is outlined in a systematic manner. Current evidence suggests that components of the immune system may be involved in the pathogenesis of atherosclerosis, with HSP acting as auto-antigens in the immune response. HSP are detected in atherosclerotic lesions and antibodies to HSP are increased in patients with vascular disease; the rise often correlating with the severity of atherosclerosis. The levels of anti-HSP antibodies have been shown to be independent predictors of risk and have prognostic value. There is a strong link between heat shock protein expression and the principal manifestations of atherosclerotic vascular diseases. A better understanding of this involvement could lead to the development of new and improved treatment strategies

Keywords: HSP; atherosclerosis; carotid disease; coronary disease; aortic disease

Abbreviations: CAD, coronary artery disease ; CP, *C. Pneumoniae* ; DM, diabetes mellitus ; HSP, heat shock proteins ; IRI, ischaemia reperfusion injury ; LDL, low-density lipoprotein ;

INTRODUCTION

More than 40 years ago, Ritossa observed that exposing larval salivary glands from Drosophila to heat, induced specific genes in the giant chromosomes of these cells (Ritossa, 1962). It is now known that these genes code for a family of proteins called

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heat shock proteins. Heat shock proteins (HSP) are a group of highly conserved proteins found in the cells of all organisms, from the simplest of prokaryotes to the most complex mammals including man (Lindquist, 1986). The term heat shock protein however is a misnomer. A better title would be "stress proteins", because in addition to raised temperature, HSP synthesis is increased in response to many environmental stresses (stress-inducible) like oxidative stress, nutritional deficiency, ultraviolet radiation, chemicals, viruses and ischaemia-reperfusion injury. Although stress-inducible, low levels of HSP expression occurs in normal physiological conditions (constitutive expression) making up 5–10% of the total protein content in healthy growth conditions (Pockley, 2003; Whitley et al., 1999).

HSP function as molecular chaperones guiding newly formed polypeptides through folding/unfolding steps to achieve correct functional configuration (Beckmann et al., 1990). They are also involved in protein transport across intracellular membranes and the repair of denatured proteins (Figure 1). HSP are grouped into various families according to their molecular weight: namely the 110, 90, 70, 60, 40 kDa and low molecular weight families. A selection of the more important members of the HSP family is detailed in Table 1. HSP have been implicated in the pathogenesis of several disease processes; however, their role in atherosclerosis particularly is widely studied. There are two main reasons for the widespread research in the link between HSP and atherosclerosis. Firstly they represent the cell's response to

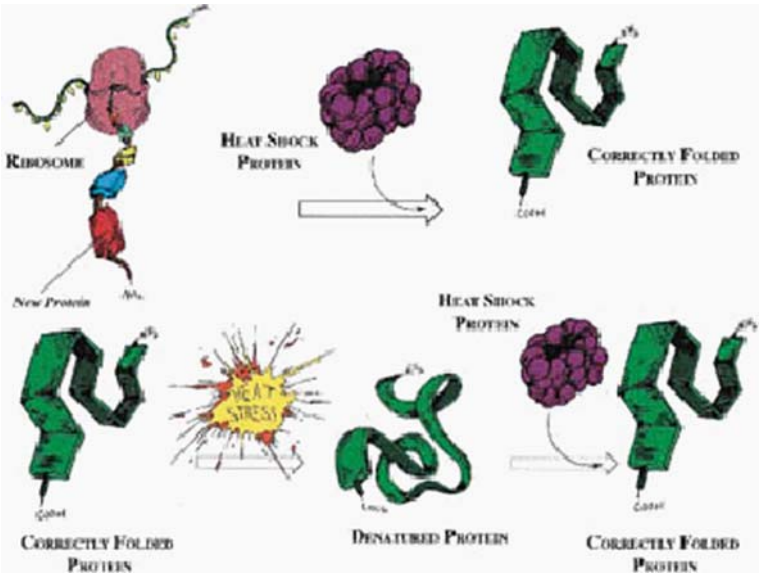

Figure 1. Two functions of heat shock proteins. *Top:* As new polypeptide chains (proteins) are being produced by ribosome within the cell, heat shock proteins assist in correct folding of polypeptide chain into functional protein. Presence of heat shock protein (*purple*) assures that the new protein will assume its functional three-dimensional configuration. *Bottom*: After stress event, heat shock proteins also assist in refolding or degradation of damaged or denatured proteins (Whitley et al., 1999).

Table 1. Key members of the heat shock protein family in humans[1]

HSP member	Location	Description
Small HSP		
Ubiquitin	Cytoplasm/nucleus	Facilitates targeting and removal of denatured proteins
Hsp10	Mitochondria	Cofactor for HSP60
Hsp27	Cytoplasm/nucleus	Involved in intracellular actin dynamics
β-crystallin	Cytoplasm	Involved in cytoskeletal stabilisation
HSP40		
Hsp40	Cytoplasm/nucleus	Regulates activity of HSP70, binds non-native protein
Hsp47	Endoplasmic reticulum	Processing of pro-collagen
HSP60		
Hsp60	Mitochondria	Molecular chaperone
HSP70		
Hsp72	Cytoplasm/nucleus	Highly stress inducible, protects against ischemia
Hsp73	Cytoplasm/nucleus	Constitutively expressed molecular chaperone
Hsp75	Mitochondria	Induced by stress including hypoxia
HSP90		
Hsp90	Cytoplasm (migrates to nucleus)	Part of the steroid receptor complex
HSP110		
Hsp110	Nucleolus/cytoplasm	Thermal tolerance
Hsp105	Cytoplasm	Protein refolding

[1] Data derived from (Pockley, 2003; Whitley et al., 1999).

stress, in particular the stress caused by risk factors of atherosclerosis and secondly they are involved in immune responses that initiate and perpetuate the inflammatory process in atherosclerotic vascular disease (Wick, 2006). The aim of this review is to highlight the link between HSP and atherosclerotic vascular disease.

Literature relevant to this review was identified by a systematic search in the Medline (Pub Med) database from 1960 onwards. HSP, vascular disease and human were used to limit the search, which was further restricted to papers in the English language. Papers dealing with in vivo studies involving the role of HSP in the pathogenesis of atherosclerotic vascular disease were accepted rather than in vitro studies in tissue and cell samples. The search was refined by careful review of references cited in key papers on HSP. For a clearer understanding of the complex role of HSP in atherosclerotic vascular disease, such diseases are classified into four main areas namely – carotid, coronary, aortic and peripheral vascular disease. After an initial overview of the pathogenesis of atherosclerosis with particular reference to HSP, the link between HSP and atherosclerosis is explored in more detail in the four areas of vascular disease.

HSP AND ATHEROSCLEROSIS

In relation to atherosclerosis, HSP from the HSP60 and HSP70 families are most widely investigated. Studies have shown that HSP60 localizes selectively in

atherosclerotic lesions as opposed to non-atherosclerotic regions of the arterial wall (Kol et al., 1998). In advanced atherosclerotic lesions, HSP70 is over expressed in several cell types, including monocytes, macrophages, dendritic cells and smooth muscle cells. In early atherosclerotic lesions however, only dendritic cells, which are key cells in the immune response, over express HSP70 (Bobryshev and Lord, 2002). Interestingly, HSP, which are normally intracellular, have also been found in a soluble form in serum along with specific anti HSP antibodies, and some studies suggest a correlation between the levels of these antibodies and the severity of atherosclerosis (Pockley et al., 1999, 1998). In addition to humoral immunity, cellular immunity has also been implicated in atherogenesis. The presence of HSP60 specific T lymphocytes in circulation may increase the risk of atherosclerosis (Ayada et al., 2007). Certain specific cellular responses to HSP60 correlated to intima-media thickness in young persons suggesting a role in very early stages of atherosclerosis (Knoflach et al., 2007). HSP have been investigated in association with many established risk factors for the development of atherosclerosis and as independent markers of the disease. In a large population based study, high levels of soluble HSP60 correlated with LDL cholesterol (Xu et al., 2000). Levels of anti-HSP 70 antibodies were significantly and independently elevated in 111 established hypertensive men as compared with 75 normotensive controls (Pockley et al., 2002). The same group also showed that HSP 70 antibodies may have a protective effect in hypertensive subjects by modifying the progression of atherosclerosis (Pockley et al., 2003). In British civil servants, an association was found between HSP60 and various psychosocial measures like low socio-economic status, social isolation and psychological distress (Lewthwaite et al., 2002). In addition to traditional risk factors for atherosclerosis, prominent immuno-reactivity against HSP60 was associated with atherosclerosis in male youngsters as measured by intima-media thickness in carotid and femoral arteries (Knoflach et al., 2003). HSP also have prognostic significance in predicting morbidity and mortality due to atherosclerosis. In a cohort of 79 men with documented coronary artery disease, significantly higher levels of anti-HSP 65 antibodies were found in those who went on to have cardiovascular events than those who did not (Hoppichler et al., 2000).

Another HSP widely studied is Haemeoxygenase-1, classified as HSP 32. Haemeoxygenase-1 is the inducible form of haemeoxygenase, an enzyme essential for haeme degradation. It is induced by a variety of stressors and performs anti-atherogenic functions in the arterial wall like scavenging reactive oxygen species, reducing monocyte adhesion and chemotaxis(Ishikawa, 2003). It is unclear whether HSP are protective or destructive for the organism and the role of HSP in atherosclerosis is most probably multifaceted. More recently, HSP27 has been studied in atherogenesis. HSP27 needs phosphorylation for its functioning and this activity was decreased step-wise from normal arteries, areas near atherosclerotic lesions and in the plaque itself. HSP27 shows promise as a diagnostic tool for early atherosclerotic disease (Wick, 2006). The causal relationship between HSP and atherosclerosis has been extrapolated in animal models such that injecting anti-HSP60 antibodies

from the sera of patients with documented atherosclerosis into the tail vein of mice accelerated atherosclerosis in them (Mandal et al., 2005).

One of the important areas of current research is to investigate the role of HSP in the association between infection and atherosclerosis. Based on their observations, Wick et al. have proposed an autoimmune hypothesis of atherogenesis. HSP60 is expressed by the endothelial cells of stressed arteries and because of sequence homology between microbial and human HSP60, the cost of immunity against micro-organisms may be responsible for endothelial cell damage and early atherosclerosis (Wick et al., 2001). This notion is explored further in relation to key areas of atherosclerotic vascular disease.

CAROTID DISEASE

Initial work in atherosclerosis and HSP focussed on carotid disease, possibly because of the ease of measuring atherosclerotic lesions in the carotid artery by ultrasound scanning. In a landmark paper, Xu and colleagues studied 867 randomly selected normal inhabitants of South Tyrol and showed that serum anti-HSP65 antibodies were significantly elevated in elderly subjects with carotid atherosclerosis. Further, a significant positive correlation was found between anti-HSP65 antibodies and the thickness of the atherosclerotic plaque in the carotid artery as measured by ultrasonography (Xu et al., 1993). Anti-HSP65 antibody titre elevation was sustained in persons with severe and progressive carotid disease and was an independent predictor of mortality at 5 years (Xu et al., 1999). Carotid atherosclerosis is an important cause of ischaemic stroke. In 180 stroke patients, anti-HSP65 and anti-HSP70 antibodies were significantly elevated 48 h after ischaemic stroke compared to controls, and multiple regression analysis showed these antibodies to be independent risk factors for stroke (Gromadzka et al., 2001). Not surprisingly although anti-HSP70 antibody level was a risk factor for ischaemic stroke, HSP70 itself may be a marker for neuro-protection in the early stages of ischaemic stroke (Jin et al., 2004). Many mechanisms have been suggested for HSP70 protection from cerebral ischaemia including defence against apoptotic and necrotic cell death (Giffard and Yenari, 2004). The biological significance of this inverse relationship between HSP70 and carotid atherosclerosis has been further investigated. A recent French study has shown that more HSP70 is released by healthy arteries than by carotid atherosclerotic plaques and this is accompanied by reduced plasma levels of HSP70 in patients with atherosclerosis compared with healthy individuals. The same study showed that levels of elastase (which can proteolyse HSP70) and markers of polymorphonuclear neutrophil activation were increased in patients with atherosclerosis and postulated that activated neutrophils could play a major role as a source of proteases able to degrade atheroprotective HSP70 (Martin-Ventura et al., 2007).

Other heat shock proteins have also been studied in relation to carotid atherosclerosis. One study has investigated the role of HSP90 as a candidate auto-antigen

in carotid atherosclerosis. This study has shown the presence of anti-HSP90 antibodies and T-cells directed against HSP90 in patients with carotid atherosclerotic plaques (Rigano et al., 2007). Recent research has focussed on HSP27. It was studied in myocardial protection models and is known to have an anti-apoptotic effect. It is the phosphorylated form of HSP27 that offers athero-protection and vessel wall cells that have a lower ratio of phosphor-HSP27 to total HSP27 may be more susceptible to oxidative stress and subsequent inflammation. This ratio was shown to be lower in normal looking artery adjacent to a carotid atherosclerotic plaque than a reference artery distant from the site of atherosclerosis. The plaque core areas showed markedly reduced levels of HSP27 and phospho-HSP27. This suggests that the apparently normal looking area near an atherosclerotic plaque is not normal at all and is highly susceptible to oxidative stress and may subsequently undergo inflammatory processes associated with early atherosclerosis (Park et al., 2006).

A large prospective follow-up study measured serum HSP60 levels 5 years apart in patients with carotid atherosclerosis and showed a consistently high correlation over a 5 year period. High serum HSP60 levels were associated with early carotid atherosclerosis and the risk of early atherosclerosis was further amplified in the presence of chronic infection. This suggests that HSP60 may be involved in activating pro-inflammatory processes associated with the early atherosclerotic lesion in the vessel wall (Xiao et al., 2005). Anti-HSP antibodies maybe produced in response to infection and this possibly provides a link between atherosclerosis and chronic infection. Infection with *Chlamydia pneumoniae* seems a likely suspect. Anti-*C pneumoniae* antibodies are most closely associated with carotid atherosclerosis as opposed to antibodies to other infectious agents. This association is independent of other risk factors, consistent over time and for different stages of atherosclerosis. The antibody titre correlates with intima-media thickness and with anti-HSP65 antibodies. Anti-*C pneumoniae* antibodies may be produced by sub-clinical infection, but interestingly evidence of chronic *C. pneumoniae* respiratory infection was more effective in predicting atherosclerotic carotid disease (Mayr et al., 2000). In addition to humoral factors, cell-mediated immunity to *C. pneumoniae* has also been associated with the pathogenesis of atherosclerosis. Established T-cell lines were propagated from activated T-lymphocytes isolated from tissue specimens of patients undergoing carotid endarterectomy. Forty-one percent of the propagated T-cell lines reacted with *C. pneumoniae* peptides and majority of these were from the HSP60 antigen (Mosorin et al., 2000). In another study, *C. pneumoniae* was present in the endothelial cells, activated macrophages and smooth muscle cells in 90% of the specimens harvested at carotid endarterectomy. *Chlamydial* HSP60 was found in all specimens positive for *C. pneumoniae*-specific antigen and mainly co-localised with this antigen in the activated macrophages, suggesting the role of *Chlamydia*-infected macrophages in the pathogenesis of carotid atherosclerosis (Kuroda et al., 2003). In summary, microbial infection causes immune reactions involving HSP via humoral and cell-mediated immune systems. Since HSP are preserved with similar antigenic properties across different species, antibodies and stimulated T cells

may then cross-react with host endothelial cells expressing such molecules perhaps promoting atherogenesis.

CORONARY DISEASE

Ischaemic heart disease is the most common cause of death in the Western world and extensive studies on HSP and coronary atherosclerotic disease have been performed. The relationship between HSP and most aspects of ischaemic heart disease has been studied including presence and severity of coronary artery atherosclerosis, coronary syndromes, myocardial infarction (MI), ischaemia-reperfusion injury and cardiac protection. Diabetes Mellitus (DM) is an important risk factor for coronary artery disease (CAD). In the last decade the incidence of diabetes has increased mainly due to obesity and its relationship to type II diabetes. There are also racial differences in the incidence of DM and CAD. A large British study analysed HSP60 levels in the plasma of 855 diabetic patients. Levels of HSP60 were higher in Caucasians than other ethnic groups. Levels of HSP60 were also higher in diabetics with manifest cardiovascular disease particularly a history of MI (Shamaei-Tousi et al., 2006). In patients with coronary artery disease (CAD), antibodies to both HSP65 and HSP60 have been shown to be significantly associated with both presence and severity of the disease. Higher titres were found with increasing number of diseased vessels and also greater extent of disease, as measured by coronary atherosclerosis scores (Birnie et al., 1998; Zhu et al., 2001). In a recent large study antibodies to HSP60 were measured in over 1000 patients with coronary heart disease and a similar number of age and sex matched controls. The concentration of anti-HSP60 was significantly higher in patients than controls. Furthermore increasing concentrations of anti-HSP60 were associated with an increased risk of coronary heart disease and with increasing severity of disease. The risk of coronary heart disease with high anti-HSP60 titres was further increased in the presence of hypertension and diabetes. The study concluded that anti-HSP60 is independently associated with coronary heart disease risk and the combination of high anti-HSP60, hypertension and diabetes is particularly detrimental (Zhang et al., 2008). In order to establish a true correlation between anti-HSP60 and CAD, the levels of these antibodies should fall following treatment. This was shown by a recent Korean study. Thirty nine patients with established CAD who had undergone percutaneous coronary revascularisation were treated with a statin and 14 weeks of cardiac rehabilitation therapy. There was a significant fall in the titres of anti-HSP60 antibodies in these patients at the end of treatment, which was accompanied by improved levels of lipids, C-reactive protein and interleukin-6 (Shin et al., 2006). Another closely studied HSP is HSP65. Anti-HSP65 antibody, although elevated in patients with CAD, significantly fell in patients following acute MI. It is hypothesized, based on animal models of MI that HSP are released in the circulation from the infarcted heart tissue and these bind to circulating anti-HSP antibodies to form antigen-antibody complexes, which are subsequently removed from the circulation by the reticuloendothelial system (Hoppichler et al., 1996).

In addition, following percutaneous transluminal coronary angioplasty, anti-HSP65 antibody titres dropped in patients who remained disease-free, but remained elevated in those who developed re-stenosis. A fall in HSP antibody titre maybe associated with a favourable outcome, and may serve as a useful prognostic marker for coronary angioplasty (Mukherjee et al., 1996). Levels of HSP60 and HSP65 are associated with more severe forms of CAD, but some other HSP may have a protective effect. High levels of human HSP70 were shown to be associated with low CAD risk, suggesting a more complex role for these proteins in coronary atherosclerosis (Zhu et al., 2003). Correspondingly, serum levels of anti-HSP70 antibodies were significantly lower in patients with stable and unstable angina compared with controls. These levels were no different in patients with stable and unstable angina and remained low during follow-up (Herz et al., 2006). Recent studies have suggested that HSP70 may serve as a "damage signal" for the immune system and could be the endogenous ligand for toll-like receptor 4 mediating synthesis of inflammatory cytokines. In a Japanese study circulating levels of HSP70 were higher in patients with acute myocardial infarction on day 1 and remained high on day 14 after MI. A fortnight after acute MI the levels of HSP70 were higher in patients with heart failure. These levels also correlated with monocyte toll-like receptor 4, interleukin 6 and tumour necrosis factor – alpha levels in plasma suggesting a role of signal mediated immune response for HSP70 (Satoh et al., 2006). Recent focus of HSP research in coronary atherosclerosis has concentrated on HSP27. HSP27 is highly expressed in the heart (Tallot et al., 2003), it is induced by oxidative stress and has an anti-apoptotic effect (Vander Heide, 2002). In one study, the plasma levels of HSP27 were increased in patients with acute coronary syndrome and these levels correlated with levels of HSP70 and C-reactive protein. The study suggested that HSP27 may increase in earlier stages of atherosclerosis and the observed increased plasma levels of HSP27 in patients with acute coronary syndromes may represent the vulnerable complex atherosclerotic plaque with associated increase in inflammatory or oxidative stress (Park et al., 2006). In another study, the plasma levels of anti-HSP27 IgG antibodies in 94 patients with acute coronary syndrome (MI and unstable angina) was significantly higher than controls in the first 12 h but not in the blood collected in the subsequent 12 h, suggesting that anti-HSP27 antibodies may be an early marker of myocardial ischaemia (Ghayour-Mobarhan et al., 2008).

In coronary atherosclerotic disease circumstantial evidence links infection, immunity and the atherosclerotic process with HSP, particularly infection with *C. pneumoniae*. A strong association between antibodies to the organism and coronary artery disease has long been established (Saikku et al., 1988, 1992) and *C. pneumoniae* has also been detected in atheromatous plaques (Kuo et al., 1993; Ramirez, 1996), but how *C. pneumoniae* might induce or promote atherosclerosis was not clear until recently. In surgical specimens from human atherosclerotic arteries, *Chlamydial* HSP60 and human HSP60 were found to co-localize within atherosclerotic plaque macrophages and both were shown to potently stimulate the production of tumour necrosis factor (TNF) and MMP-9 by the macrophages (Kol et

al., 1998). *Chlamydial* lipopolysaccharide has been demonstrated to induce mononuclear phagocyte foam cell formation and *Chlamydial* HSP60 was shown to induce low-density lipoprotein (LDL) oxidation (Kalayoglu et al., 2000). These steps are highly relevant to atherogenesis and plaque complications. Further evidence of this coronary atherosclerosis/*Chlamydial* infection/HSP association is suggested in other clinical studies. High levels of anti-human HSP60 antibodies and *C. pneumoniae* antibodies were found to be independent risk factors for coronary atherosclerosis, and their simultaneous presence substantially increased the risk of disease development (Burian et al., 2001; Heltai et al., 2004). Using multivariate analysis to account for other risk factors high levels of anti-*Chlamydial* HSP60 antibodies were again shown to be independently associated with coronary artery disease and could identify the subset of patients with *Chlamydial* infection and significant CAD (Mahdi et al., 2002). A persistent elevation in the immune response involving *C. pneumoniae* and HSP60, when present together, better predicted coronary events than transient or individual elevations in these antibodies (Huittinen et al., 2003). A recent study examined the risk of secondary cardiovascular event in over a thousand patients with acute manifestations of coronary heart disease. This study measured the status of *C. pneumoniae* (CP) infection, CP HSP60 and human HSP60 IgG and IgA antibodies at baseline and followed-up these patients for 33 months. Their results showed that the risk of secondary cardiovascular events was increased among patients with both a positive CP sero-status and diabetes compared to infection negative non-diabetic patients and in general sero-positivity added a hazard to diabetes. The study was not able to show a role of human HSP60 sero-status in the development of secondary cardiovascular events in patients with coronary heart disease (Guech-Ongey et al., 2006). Some other studies have failed to show the link between CP infection and coronary heart disease. When measured in 151 patients with ischaemic heart disease, antibody responses to CP IgG, human or *Chlamydial* HSP60 were not associated with endothelial dysfunction and the presence and severity of coronary artery disease (Hoymans et al., 2008). Other infectious agents have been studied in the HSP and coronary heart disease link. *Helicobacter pylori*, particularly the CagA strain of *H. pylori* has been shown to concur to the development of coronary heart disease (Lenzi et al., 2006). It has long been recognised that there is a link between gum disease and cardiovascular disease. In one study, four groups of patients were studied: patients with chronic periodontitis, patients with coronary heart disease (CHD), patients with both periodontitis and CHD and controls. T cells in the peripheral blood were primed to both microbial HSP65 and human HSP60, but CD4 proliferative responses were found only with human HSP60 and this was most significant in patients with both periodontitis and CHD (Hasan et al., 2005). In another study, the inflammatory infiltrate of atherosclerotic plaques was examined for the presence of HSP60 and periodontal bacteria using monoclonal antibodies. Human HSP60 expression was evident on endothelial cells, smooth muscle cells and lymphocytes. Four common periodontal pathogens were found in increasing number of arteries with atherosclerotic plaques. This suggests a specific immune response

associated with atherosclerosis involving common bacterial pathogens possibly via heat shock proteins (Ford et al., 2006).

There is evidence for the link between infection and atherosclerosis via heat shock proteins and this evidence has paved the way for research trials to evaluate the role of immunomodulation, antibiotics and vaccination against *C. pneumoniae* as interventional measures in CAD (Leinonen and Saikku, 2002; Zhou and Hansson, 2004) Although initial clinical studies showed promising results, these new anti-atherogenic measures have not yet had any long term impact on atherosclerosis. Possibly, this is because of wrong choice or short duration of antibiotics or the fact that the disease aetiology is multi-factorial and not simply dependent on one infectious agent (Muhlestein, 2002; Tsirpanlis, 2004). Long-term trials are required to investigate further this interesting area of research.

AORTIC DISEASE

HSP are implicated in atherosclerotic disease involving the abdominal aorta and its visceral branches. Using immuno-histochemical techniques in human aortic specimens from autopsy, HSP70 was shown to be present weakly throughout the media of apparently normal looking specimens, and was highly concentrated in the centre of thickened atheromatous plaques around sites of necrosis and lipid accumulation. The intensity of HSP70 staining correlated with the thickness of the atherosclerotic plaque. Also the increased HSP distribution was prominent in macrophages as opposed to smooth muscle cells or other plaque cells (Berberian et al., 1990). Another study investigating HSP70 in the human aorta studied its distribution by immuno-histochemistry and video image analysis software, and quantified the level of HSP70 by Western Blotting. This study showed a homogeneous staining pattern of HSP70 in "normal-appearing" regions, but a heterogeneous pattern in areas of atherosclerosis. The image analysis indicated a significant positive correlation between the severity of atherosclerosis and altered pattern of HSP70 staining, but Western blotting showed no difference in total content with plaque progression. It is suggested that the heterogeneous pattern of HSP distribution in atherosclerotic lesions may be due to leakage of HSP70 from damaged cells into the plaque (Johnson et al., 1993). Stress-induced synthesis of HSP normally protects cells from death, but insufficient accumulation of HSP70 in aortic smooth muscle cells, near areas of necrosis, leads to death of such cells, which further promotes plaque rupture and thrombo-embolic complications (Johnson et al., 1995).

Although most modern investigators question the relationship between aortic aneurysmal disease and atherosclerosis, immune responses have been studied in abdominal aortic aneurysmal disease. Serum level of anti-HSP70 antibodies was significantly higher in patients with abdominal aortic aneurysms (AAA) than controls indicating a role for humoral immune response involving HSP (Chan et al., 1999). Cell-mediated immunity is also implicated as HSP60 expression was found in intimal endothelial cells and mononuclear infiltrate (T-lymphocytes and macrophages)

of the aorta at sites of branching and other large size arteries, but not in smaller vessels. This distribution of immune cells suggests that the stress of high velocity and haemodynamic sheer forces may be responsible for the recruitment of HSP-specific T cells (Kleindienst et al., 1993). Infection may be responsible for initiating the immune responses, and once again *C. pneumoniae* has been implicated, as it was detected in the vessel wall of AAA specimens using different techniques including immuno-histochemistry, in-situ hybridisation and polymerase chain reaction. To satisfy the infection hypothesis, all components of *C. pneumoniae* should be isolated in the atherosclerotic plaque. Whilst *Chlamydial* lipopolysaccharide and membrane protein antigens were detected in abundance, *Chlamydial* DNA or HSP were not, and this was suggested to be due to the rapid degradation of the HSP and DNA with persistence of other antigens (Meijer et al., 1999).

PERIPHERAL VASCULAR DISEASE

Peripheral vascular disease (PVD) usually refers to atherosclerotic chronic lower limb ischaemia. Patients with PVD have three times higher mortality than age and sex matched controls mainly because of co-morbidity due to atherosclerotic disease in other vascular beds (Leng et al., 1996). A number of studies have analysed the role of HSP in the pathogenesis of atherosclerotic PVD and particularly the beneficial influence of exercise. Levels of circulating HSP70 were significantly elevated in twenty patients with PVD compared to controls. Levels of anti-HSP70 antibodies were also elevated but this did not reach statistical significance. In the same study, anti-HSP60 antibody levels were significantly elevated in patients with PVD and the level demonstrated positive correlation with the disease severity (Wright et al., 2000). Similarly, in another study, levels of anti-HSP70 antibodies were found to be significantly elevated in patients with PVD as compared with controls and these levels were higher in patients with critical ischaemia than in claudicants, again suggesting that anti-HSP antibody levels bear some correlation with disease severity (Chan et al., 1999). Diabetes mellitus is one of the more important risk factors for the development of PVD and anti-HSP70 antibody subclasses have been measured in 67 diabetic patients. IgG and IgM class of anti-HSP70 antibodies were not different as compared to controls, but IgA class anti-HSP70 antibodies titres were significantly higher in type I and II diabetics than in non-diabetic controls. It has been suggested that IgA-containing immune complexes may be implicated in the vascular complications of patients with diabetes mellitus and that HSP70 may have a role as an auto antigen in the pathogenesis of these diseases (Figueredo et al., 1996).

Transluminal angioplasty is a common treatment modality for patients with PVD, but the mechanical stretch injury associated with balloon angioplasty can lead to lipid accumulation, monocyte and platelet adhesion, smooth muscle cell proliferation and new plaque formation (Ip et al., 1990). Interestingly, the mechanical stress of balloon angioplasty was shown to induce HSP70 in the smooth muscle cells in of blood vessels harvested from patients undergoing above-knee amputations. On the basis

of these results it has been suggested that the induction of HSP70 expression may be an important component of the response to injury by blood vessels that is often described as the first step in atherogenesis (Kirby et al., 1999). HSP70 and HSP70 mRNA was measured in calf muscle biopsies from patients in different stages of PVD, using gel electrophoresis and reverse transcriptase polymerase chain reaction techniques respectively. Both HSP70 and HSP70 mRNA were significantly elevated in calf muscles from patients with Fontaine stage II, III and IV PVD as compared to controls. The highest levels were found in stage III disease, whereas levels were lower in stage IV disease, which is characterised by tissue loss. This was hypothesized to be due to lack of exercise in patients with stage IV disease. Exercise in patients with PVD may cause an ischaemia-reperfusion type injury. Since stage IV patients frequently have severely limited mobility and are unable to exercise, there is an absence of reperfusion injury and a reduction in HSP70 levels (Liu et al., 2002). Ischaemia reperfusion injury (IRI) has been implicated in the pathogenesis of many diseases in the heart, lung and intestines. In PVD ischaemia occurs during walking to the point of claudication and reperfusion occurs when the patient stops and rests. This IRI has been suggested to cause inflammation and atherosclerotic progression, perhaps accounting for the higher mortality seen in these patients compared to age and sex matched controls (Tisi and Shearman, 1998). Reperfusion of ischaemic tissues initiates a complex series of reactions that paradoxically injures tissues. Leukocyte-endothelial interaction is a pivotal step in this IRI. Thermo tolerance, possibly mediated through the induction of HSP72, attenuates ischaemia-reperfusion induced leukocyte-endothelial interaction, the key process in IRI (Chen et al., 1997).

In healthy individuals, a bout of acute exercise induces HSP. Serum HSP70 levels were elevated during and after a 60-min treadmill exercise in normal humans (Walsh et al., 2001). Although exercise increases the body temperature, exercise-induced hyperthermia is not the sole factor inducing HSP70 production; this is likely to be a combination of mechanical, metabolic and chemical stresses. It is inferred that this induction of HSP may play a role in the adaptation to exercise and training (Kilgore et al., 1998). The role of HSP in exercise in patients with PVD merits further investigation.

CONCLUSION

There is growing evidence that atherosclerosis may be an inflammatory and possibly an immune disorder. Due to a high degree of sequence homology between human and microbial HSP, anti-microbial HSP antibodies produced in response to infection cross-react with HSP on endothelial cells stressed by classical risk factors for atherogenesis. This “antigenic mimicry” causes endothelial damage and early atherosclerotic lesions (Wick et al., 2004; Mandal et al., 2004). In each of the four key areas of vascular disease – carotid, coronary, aortic and peripheral vascular disease – studies have shown a substantial role of HSP in the pathogenesis of these diseases. In many cases, they are independent risk factors for the disease. Levels of HSP or their specific antibodies have a positive correlation with disease severity and

in some cases have prognostic value. Although for the individual cell, HSP are helpful for cell survival as they perform important functions, it is not clear that for the organism as a whole, HSP are beneficial or simply a double-edged sword playing a role in the pathogenesis of fatal diseases. A more comprehensive understanding of the role of HSP in atherosclerosis is likely to lead to new approaches for the prevention and treatment for all forms of cardiovascular disease.

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CHAPTER 7

HEAT SHOCK PROTEINS AND CANCER

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Abstract: Heat shock proteins (HSP) play multiple roles in cellular physiology and pathology depending on a wide variety of factors including its relative location within the cell (intracellular, plasma membrane or extracellular milieu), the age of the cell or whether it has undergone neoplastic transformation. In normal non-transformed cells, HSP play a cytoprotective role and protect cells from adverse stressful stimuli via chaperoning naïve, misfolded and/or denatured proteins by a process known as the stress response. However, cancer cells have commandeered this function and the result is increased resistance to a number of anti-cancer therapies including hyperthermia, radiation and a wide range of chemotherapeutic agents. Recent advances in our understanding of this dual role of HSP have led to the development of pharmacological and molecular tool to target HSP for therapeutic gain. In this chapter, we highlight evidence for the involvement of HSP in the pathology of various cancers and discuss their proposed mechanism of action and therapeutic potential

Keywords: Apoptosis; cancer; cytotoxicity; heat shock proteins; tumors

Abbreviations: AP-1, activator protein-1; AR, androgen receptor; CRC, colorectal cancer; CTL, cytotoxic T lymphocytes; ERK, extracellular-signal regulated kinase; GRP, glucose regulated proteins; HBV, hepatitis B virus; HCC, hepatocellular carcinoma cells; HCV, hepatitis C virus; HER, human endothelial growth factor receptor; HSF, heat shock factor; Hsp, heat shock proteins; *hsp*, heat shock protein gene; HSP, heat shock protein family; *HSP*, heat shock protein family gene; JAK/STAT, janus-activated-kinase/signal transducer and activator of transcription; MAPK, mitogen activated protein kinase; NK cells, natural killer cells; NSCLC, non-small cell lung cancer; PSA, prostate specific antigen; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; SCC, squamous cell carcinoma; SCLC, small cell lung cancer

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INTRODUCTION

Heat shock proteins (HSP) were first discovered in 1962 as a set of highly conserved proteins found in eukaryotes and prokaryotes that were induced by hypethermia and other kinds of cellular insults (Ritossa, 1996). HSP are ubiquitous proteins and were initially characterized as cytoprotective molecular chaperones. The important function of a molecular chaperone is to assist a protein to attain functional conformation, to mediate interaction with other proteins and to prevent non specific aggregation of misfolded proteins (Ellis and van der Vies, 1991; Georgopoulos and Welch, 1993; Lindquist and Craig, 1988). Molecular chaperones facilitate a range of processes including protein folding, transport of proteins across membranes, modulation of protein activity, regulation of protein degradation and prevention of irreversible protein aggregation. The latter activity is believed to be critical to survival of high temperature stress (Lindquist, 1986). Mammalian HSP have been classified into groups according to their electrophoretic mobility. The small HSP families include alpha crystallin, Hsp10, Hsp27 and Hsp40, whereas, the large HSP families include Hsp60, Hsp70, Hsp90 and Hsp170. The large HSP families are ATP dependent molecular chaperones and small HSP function in an ATP independent manner and are important for signaling and protein traffic even in the absence of stress and regulated by specific heat shock transcription factors (Mosser and Morimoto, 2004). However, the need of HSP increases markedly after a cellular assault as a defense mechanism to allow cells to survive otherwise lethal conditions. Hsp gene transcription is regulated by heat shock factor (HSF) that ensure prompt transcriptional activation in stress and it will switch-off after recovery (Sorger and Pelham, 1988).

There is cascade of molecular events that mediate the transformation of normal cell into a cancer cell. Several etiological agents and factors have been identified as responsible for initiating the neoplastic transformation process, including are viruses, radiation, carcinogenic compounds, and hereditary and non-hereditary genetic mutations. Most tumors are formed by stepwise progression of normal cells into a transformed tumor cells by using alterations in cell physiology including self sufficiency in growth signals, insensitivity to growth inhibition, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Heat shock response participates in tumor development usually by up-regulation of but also by down-regulation of specific HSP. It is well known that variations in HSP expression can be found in pre-neoplastic and neoplastic human tumors in different tissues and organs. At the histopathological level, in most cases the transition from a normal tissue to a cancer tissue is accompanied by an increase in HSP expression. HSP are involved in the tumor development and protect cells from apoptosis and induce drug resistance. In this chapter, we highlight evidence for the involvement of HSP in the pathology of various cancers and discuss their proposed mechanism of action and therapeutic potential.

BREAST CANCER

Breast cancer is the most common cause of cancer in women and the second most common cause of cancer death in women in the USA. It is a disease with complex etiology, that is influenced by many factors which include genetic, biologic, lifestyle and environment (Cleator and Ashworth, 2004; Veronesi et al., 2005). However the precise mechanisms which mediates cellular transformation during tumorigenesis, maintain the malignant phenotypes and allow cells to migrate to distant organs as a process called metastasis are poorly understood. Data emerging from several studies indicates that complex changes in the expression and/or function of specific cellular proteins contribute to the development and progression of cancers by conferring growth advantages and survival properties to tumor cells. HSP expression levels are known to be elevated in a number of cancers including breast cancer and often contribute to changes in the properties of tumor cells either during transformation, progression or in response in drug treatment.

In breast cancer, three HSP families are found to be significantly elevated including the Hsp90, Hsp70 and Hsp27 chaperone families. The Hsp90α protein is commonly referred to as the cancer chaperone (Neckers, 2007) and shown to play an important role in the essential signal transduction pathways regulating proliferation, differentiation, apoptosis, angiogenesis, metastasis, oncogenesis (Cullinan and Whitesell, 2006), genetic variation (Queitsch et al., 2002), invasion (Eustace et al., 2004) and cellular transformation (Teng et al., 2004). Perotti and coworkers used subtractive hybridization to identify a number of prolactin-regulated genes in the human mammary carcinoma cell line SKBR3. Northern blotting analysis and luciferase assays identified the gene encoding *HSP90A* as a prolactin-JAK2-STAT5 target gene (Perotti et al., 2008). These authors concluded that elevated expression of *Hsp90A* in breast cancer is correlated with increased cell survival and poor prognosis (Perotti et al., 2008). In a separate study Pick and colleagues examined tissue microarrays containing 10 cell lines and primary specimens from 655 patients with 10-year follow-up using an automated quantitative analysis (AQUA) method and similarly assessed estrogen receptor, progesterone receptor, and Her2/neu expression. Hsp90 expression was found to be more variable in human tumors than in cell lines. High Hsp90 expression was associated with decreased survival. On multivariable analysis, high Hsp90 expression remained an independent prognostic marker and was associated with high Her2/neu and estrogen receptor, large tumors, high nuclear grade, and lymph node involvement. Although Hsp90 levels were high in all cell lines studied, the expression in primary tumors was more variable. The authors concluded that high Hsp90 expression in primary breast cancer defines a population of patients with decreased survival (Pick et al., 2007). Yano et al. examined the expression of Hsp90α, Hsp90β and cyclin D1 in human breast cancer. Levels of mRNAs coding for *hsp90α* and *cyclin D1* were significantly higher in cancer tissues than in non-cancer tissues. Moreover, there was a close relationship between the extents of the

two mRNA levels, suggesting that increased expression of *hsp90α*, an isoform of the HSP90 family, is associated with the proliferation of human breast cancer. Hsp90β was expressed in cancer cells, but not associated with cell proliferation (Yano et al., 1999).

Hsp70 is a major inducible chaperone and is present at elevated levels in various tumors, especially of epithelial origin including breast cancer. The expression of Hsp70 in breast tumors often correlates with increased cell proliferation, lymph node metastases, poor response to chemotherapy and poor survival (Calderwood et al., 2006; Ciocca and Calderwood, 2005; Jaattela, 1999). In a clinical study, Kalogeraki et al. evaluated the relationship between the expression of Hsp70 protein, cell proliferation, the expression of estrogen receptors (ER) and the clinicopathological variables Grade and LNS in breast invasive human tumors along with the role of Hsp70 protein in the prognosis of human breast cancer (Kalogeraki et al., 2007). A strong association between Hsp70 expression and ER content was found which revealed a statistically significant association between Hsp70 positivity and ER expression in 50 cases of invasive primary human breast cancers. These authors also found a strong correlation between Hsp70 expression, Grade and LNS of invasive ductal breast carcinomas. This suggests that the expression of Hsp70 plays a significant role in the progression of human breast cancer. In a separate study the overexpression of Hsp70 in human breast cancer MCF-7 cells resulted in strong acceleration of cell growth by shortening of G0/G1 phases (Barnes et al., 2001). This effect could be related to stabilization of cyclin D1 upon production of Hsp70 (Diehl et al., 2003). Nylandsted et al. studied the role of Hsp70 in human tumor cells and concluded that Hsp70 confer survival advantages to tumor cells. These authors generated an adenovirus expressing antisense Hsp70 (Ad.asHsp70). The effective and specific depletion of Hsp70 by Ad.asHsp70 resulted in massive cell death of all tumorigenic cell lines. Anti-apoptotic proteins Bcl-2, Bcl-$X_L$and CrmA as well as peptide-inhibitors of caspases, DEVD-CHO and zVAD-FMK, failed to rescue tumor cells from Ad.asHsp70-induced cell death. These results indicate that the high expression of Hsp70 is a prerequisite for the survival of human cancer cells of various origins and reveal Hsp70 as the only protein described so far whose expression is specifically required for the survival of neoplastically transformed cells (Nylandsted et al., 2000).

The small heat shock protein, Hsp27, is elevated in a significant proportion of breast cancer cell lines, compared with the lower levels found in normal breast tissue and benign breast lesions (Ciocca and Calderwood, 2005). Hsp27 regulates the stability and cytoskeleton organization of cells and the overexpression of Hsp27 in breast cancer cells increases anchorage independent growth, invasiveness and resistance to chemotherapeutic drugs including doxorubicin and cisplatin and associated with poor prognosis. Storm et al. investigated the distribution of Hsp27 in noncomedo and comedo DCIS, and DCIS associated with IDC, was evaluated by immunohistochemistry and compared with HER-2/neu expression within the same cancers. Hsp27 was overexpressed in 28 of 47 (approximately 60%) cases of DCIS; expression in pure DCIS was 16 of 24 (67%), and 12 of 23 (approximately 50%) in

DCIS associated with IDC (Storm et al., 1995). Interestingly, analyses of biopsies taken from lymph node metastasis showed an even higher percentage with elevated Hsp27 (detected in >70% of patients of metastasis) (Storm et al., 1996). Bausero et al. investigated the surface expression of Hsp27 in 4T1 murine mammary cell line and studied its role in growth and metastasis of tumors. These authors demonstrated that 4T1 mammary adenocarcinoma cells sorted for high Hsp25 surface expression (Hsp25(high)) grow significantly faster than cells sorted for intermediate Hsp25 surface expression (Hsp25(intermediate)) or wild-type 4T1 cells, when implanted into the abdominal breast gland of female BALB/c mice. In addition, histological examination of lung tissues revealed that Hsp25(high) 4T1 cells metastasized to the lungs more aggressively than either Hsp25(intermediate) or wild-type 4T1 cells (Bausero et al., 2004). These authors further showed that silencing Hsp25 in 4T1 cells abrogates the migration potential of tumor cells in vitro (Bausero et al., 2005). Nagaraja et al. recently demonstrated that lentivirus-mediated permanent silencing of Hsp25 in 4T1 mammary cells induced the regression of established tumors in mice by a mechanism dependent on the proteasome complex and mediated by $CD8^+$ cytotoxic T lymphocytes (Nagaraja et al., in preparation).

LIVER CANCER

Hepatocellular carcinoma (HCC) is cancer that arises from hepatocytes, the major cell type found in the human liver, and is the most common primary malignant tumor of the liver. Primary liver cancer accounts for less than 1% of all cancers in this country. It is the seventh most common cause of cancer related deaths in men and the ninth in women. However, the incidence in the United States has increased during the past two decades possibly due to a large pool of people with longstanding hepatitis C. Hsp70 is the major heat inducible molecular chaperone has been detected on the cell surface of tumor cells but not on normal cells (Multhoff et al., 1995). This unusual Hsp70 expression on surface of plasma membrane correlates with an increased sensitivity to human natural killer (NK) cells and might be of clinical relevance. Hsp70 plasma membrane expression was found on freshly isolated human biopsy material of liver metastases (Hantschel et al., 2000). Joo and coworkers investigated the expression of Hsp70 and Hsp27 in HCC in association to tumor cell proliferation and apoptosis. These authors examined the expressions of Hsp70 and Hsp27 by immunohistochemical staining in 71 cases of HCC, and then related the expression to clinicopathologic parameters and expressions of p53, Ki-67 and Apotag. Hsp70 and Hsp27 were frequently stained in the cytoplasm and nuclei of tumor cells, but not in the non-neoplastic hepatocytes. Immunoreactivities of Hsp70 and Hsp27 were observed in 56.3 and 61.9% of HCC, respectively. Hsp70 immunoreactivity correlated with high Ki-67 labeling indices (LIs), large tumor size, presence of portal vein invasion, and high tumor stage. These authors concluded that expressions of Hsp70 and Hsp27 may play an important role in hepatocarcinogenesis, and especially Hsp70 showed a close relationship to the pathological

parameters associated with tumor progression and high Ki-67 LIs (Joo et al., 2005). King and colleagues investigated Hsp27 expression in patients with HCC and examined its prognostic significance in 58 HCC and adjacent noncancerous liver tissues by immunohistochemical staining. Of the 58 HCC tissues studied, the presence of Hsp27 was demonstrated in 45 tissues, low expression was demonstrated in 17 tissues and high expression was demonstrated in 28 tissues, and observed a significantly higher distribution of Hsp27 expression in HCC tissues compared with adjacent noncancerous liver tissues was obtained. Patients with high Hsp27 expression had a significantly higher histologic tumor grade than those with low Hsp27 expression (King et al., 2000).

Sun et al. investigated the proteins involved in HCC carcinogenesis and employed two-dimensional fluorescence DIGE to study the differentially expressed proteins in tumor and adjacent nontumor tissue samples. Samples from 12 hepatitis B virus-associated HCC patients were analyzed. These authors found that members of the Hsp70 and Hsp90 families were simultaneously up-regulated in HCC samples (Sun et al., 2007). Takashima et al. identified proteins linked to the pathogenesis of HCC associated with hepatitis C virus (HCV). By performing two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry these authors identified differentially expressed protein in HCC, including significant up-regulation of GRP78, GRP75 and Hsp70.1 in cancerous tissues and concluded that these four HSP play important roles in the pathogenesis of HCV-related HCC (Takashima et al., 2003). Matsushima-Nishiwaki and colleagues studied the role of Hsp27 in human hepatocellular carcinoma cell lines. The authors further showed that phosphorylated Hsp27 interferes with cell growth of the hepatocellular carcinoma derived HuH7 cells in the presence of the proinflammatory cytokine, tumor necrosis factor-α, via inhibition of the sustained activation of the extracellularsignal-regulated kinase signal pathway. The activities of Raf/extracellular-signalregulated kinase and subsequent activator protein-1 transactivation, and the induction levels of cyclin D1 were lower in HuH7 cells transfected with phosphorylated Hsp27 than those with non-phosphorylated Hsp27. Since, the ERK signal pathway is a major proliferation signal of hepatocellular carcinoma, and AP-1 activation is an early event in hepatocarcinogenesis (Matsushima-Nishiwaki et al., 2008).

In a recent study, Yao et al. reported that Hsp90 is required for the activity of hepatitis B virus (HBV) reverse transcriptase. The gp96 expression was demonstrated to be increased as the HBV-induced disease progresses from chronic hepatitis to cirrhosis, then HCC. The increased expression of gp96 which is known to be closely related to cell survival and prevention of apoptosis is suggested to account for the longevity of cirrhotic and HCC cells compared to normal hepatocytes. In support of this gp96 was strongly expressed in HCC (73.3%) and weakly in non-cancerous tissues. The gp96 expression in HCC tissues was correlated with degree of tumor differentiation and tumor size, but not with tumor number. Immunohistochemical analysis showed that 90% of HCC patients with HBV-DNA-positive strongly expressed gp96, whereas only 46% of HBV-DNA-negative patients were positive for gp96 (Yao

et al., 2006). Chuma et al. studied the expression profiles among 7 early components and 7 progressed components of nodule-in-nodule-type HCC and their corresponding noncancerous liver tissues using oligonucleotide array, and identified that the *hsp70* gene was amongst the most abundantly up-regulated gene in early HCC. RT-PCR and immunohistochemical examination confirmed that Hsp70 significantly overexpressed in early HCC compared with precancerous lesions (Chuma et al., 2003).

COLON CANCER

Colorectal cancer (CRC) is a heterogeneous neoplasm made up of cancer cells with diverse growth rates and metastatic potential (Fidler, 1990). This is presumed to be one of the reasons why the clinical behavior may be different among patients classified in the same pathological or clinical stage. Multiple proto-oncogenes, oncogenes, regulatory factors, and tumor suppressor genes have been suggested to play a role in the progression of colorectal tumors (Fidler, 1990). Many malignant solid tumors including colorectal tumors contain significant fractions of hypoxic cells as a consequence of the inadequate vascular networks (Vaupel et al., 1989). The presence of hypoxic cells in solid tumors has long been considered a problem in cancer treatment. Hypoxic tumor cells have been shown to be more resistant to radiotherapy and many conventional chemotherapeutic agents than their normoxic counterparts (Brizel et al., 1996).

Capello et al. reported that the small HSP, Hsp10 is overexpressed in early large bowel carcinogenesis. Using both immunohistochemistry and Western blot analysis these authors further demonstrated that an additional mitochondrial chaperone, Hsp60, is also overexpressed in colon cancer tissues. Immunohistochemistry results showed the presence of Hsp60 positive in all tubular adenomas and infiltrating adenocarcinomas and normal tissues and hyperplastic polyps were negative. These authors concluded that Hsp10 and Hsp60 molecular chaperones plays a different role in colorectal carcinogenesis with respect to that in normal cells, which could play an important role in apoptosis (Cappello et al., 2003a, b). Hwang et al. analyzed HSP family proteins in weakly and highly metastatic human colorectal cancer (CRC) cell lines. These authors found that expression of Hsp70 and Hsp110 was elevated in highly metastatic CX-1 and HT-29 cells. Examination of HSP expression by immunohistochemical analysis of 81 primary human CRC tissues demonstrated that the expression of Hsp70 and Hsp110 was highly correlated with the advanced clinical stages and positive lymph node involvement. It has been demonstrated that Hsp70 play important role in apoptosis by preventing the release of cytochrome C from the mitochondria and/or it may block the SAPK/JNK activation pathway and prevent apoptosis in response to external stimuli (Hwang et al., 2003). Hsp70 is the major inducible chaperone expressed on the plasma membrane of freshly isolated biopsy material from CRC patients. The presence of Hsp70 on the plasma membrane of CRC cells has been suggested to be of clinical importance as an immune recognition structure (Hantschel et al., 2000).

LUNG CANCER

Cancer arises from a complex combination of genetic and epigenetic abnormalities. Lung cancer is the leading cause of cancer-related deaths worldwide. This disease is classified into two major histological groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Tobacco smoke is a major etiological factor, especially in SCLC. Small cell lung cancer comprises approximately 20% of all lung cancers and exhibits a neuroendocrine phenotype while NSCLC lacks these features and makes up the remaining 80% of cases. SCLC exhibits a more aggressive phenotype that inevitably reoccurs after initial response to chemotherapy while the clinical outcome of NSCLC is often hard to determine (Kurup and Hanna, 2004; Zakowski, 2003). Bonay et al. studied the abundance and distribution of HSP in the normal lung, the effect of cigarette smoking on their expression, or their expression in human lung carcinomas. These authors used monoclonal antibodies coupled with immunohistochemical and immunoelectrophoretic techniques. In lung tissue from nonsmokers, bronchiolar epithelial cells were intensely positive for Hsp90 and the inducible Hsp72, but only weak reactive for Hsp63. It was also observed that macrophages expressed these HSP albeit at low levels. However, no other parenchymal or immune/inflammatory cells were positive for these HSP. Cigarette smoking did not modify the distribution or the intensity of HSP in bronchiolar epithelial cells, and macrophages from smokers expressed similar or lower levels of these HSP. Tumor cells from 14 of 15 lung carcinomas expressed one or more of the HSP. Considerable heterogeneity in the expression of HSP by cells in a given tumor was observed, explained in part by differences in the differentiation stage of the cells (Bonay et al., 1994).

The Hsp90 chaperone is required for the conformational maturation and stability of multiple oncogenic kinases that drive signal transduction and proliferation of lung cancer cells. Hsp90 plays a unique anti-apoptotic role in SCLC cells. The Hsp90 has a critical role in malignant transformation by maintaining the functional conformations of mutant and aberrant oncoproteins (Rodina et al., 2007). In a study by Volm et al. the association between the Hsp70 and drug resistance was analyzed. Tumor samples of 90 patient's NSCLC were examined using immunohistochemistry and no association between resistance to doxorubicin and Hsp70 was found. These authors further reported that of 63 resistant tumors, 33 showed low and 30 high Hsp70 expressions. Of the 26 sensitive tumors, 11 had low and 16 had high Hsp70 expression. An interesting trend was observed, in that, tumors with high glutathione S-transferase-x expression showed high Hsp70 expression. In addition, there was a significantly strong correlation between Hsp70 and catalase positivity. These data indicate that heat shock and stress promote intracellular oxidative damage and catalase is necessary for protection (Volm et al., 1995). Malusecka et al. studied the expression pattern of the *hsp70* and the *hsp27* genes in 106 cases of NSCLC. These authors found that majority of cases (95/106) the Hsp70 immunoreactivity was localized both in the cytoplasm and the nuclei, and that there was an enhanced nuclear immunoreaction for Hsp70 in dysplastic lesions and in stage I tumors. In the case

of the Hsp27, they found a positive cytoplasmic immunostaining in 70% of cases, with the highest score in squamous cell carcinoma (SCC). The results showed that positive correlation between the expression level of Hsp27 and Hsp70. The results indicates that Hsp70 and Hsp27 participates in lung tumor cell survival and these HSP are involved in resistance to chemotherapeutic drugs (Malusecka et al., 2001). In a separate study the relationship between the expression of GRP94 at the level of mRNA and protein in human lung cancer was investigated. Fifty-four cases of lung cancer tissues was analyzed and corresponding normal lung tissues by using RT-PCR, immunohistochemistry and/or Western blot techniques, and observed that there was a significant overexpression of GRP94 mRNA and protein in lung cancer tissues as compared with normal lung tissues. Furthermore, the over-expression of GRP94 in the lung cancer tissues was correlated to grade of differentiation and stage of tumors. There was stronger expression in poor-differentiated tumors than in mild-to-high differentiated tumors. There was also a stronger expression in stage III than in stage I and II tumors, but no statistically significant difference was found among various pathological types of tumors. The results indicate that GRP94 is involved with the occurrence, differentiation and progress of human lung cancer (Wang et al., 2002). Recently, Jackson and Garcia-Rojas investigated the role of Hsp27 in cellular resistance to oxidant stress in human lung cells, and reported that Hsp27, which is phosphorylated by MK2 in the MAPK pathway, protects epithelial cells from oxidant stress (Jackson and Garcia-Rojas, 2008).

PROSTATE CANCER

Prostate cancer is the most common cancer and the third most common cause of cancer related mortality in men in the United States (Jemal et al., 2006). While early detection has increased with the advent of serum prostate specific antigen (PSA) testing, the disease is often advanced when patients present with symptoms. The ability of prostate cancer cells to mount resistance to several chemotherapeutic drugs is thought to occur through several mechanisms including adaptive upregulation of anti-apoptotic genes and utilization of alternative growth factor pathways (Craft et al., 1999; Gimenez-Bonafe et al., 2004), as well as ligand-independent androgen receptor (AR) transactivation (Sherwood et al., 1998). Elevated levels of HSP have been observed in prostate cancer cells and their role is just beginning to be understood and includes inducing anti-apoptotic genes and provides cytoprotection by inhibiting cell death. Recent immunohistochemical studies using human prostate tissue have shown an association between HSP and prostate cancer aggressiveness, progression, and development of a hormone-refractory phenotype. In a clinical investigation, Cornford et al. analyzed the expression of Hsp27, Hsp60, and Hsp70 in formalin-fixed, paraffin wax-embedded, archival tissue specimens of early prostatic adenocarcinomas removed at radical prostatectomy and in advanced cancers and control prostates and also from prostate tumor cell lines. In advanced cancers, Hsp27 expression was invariably associated with poor clinical outcome

and observed elevated Hsp27 expression only in aggressive malignant cell lines and androgen-insensitive cell lines. Increased Hsp27 expression in hormone-refractory prostate cancer suggests that Hsp27 may confer resistance to androgen withdrawal by blocking apoptotic signals from androgen ablation. Expression of Hsp60 was significantly increased in both early and advanced prostate cancer when compared with nonneoplastic prostatic epithelium. The expression of Hsp70 was unaltered in early prostate cancers when compared with nonneoplastic prostatic epithelium but showed a diminished expression in morphologically advanced cancers (Cornford et al., 2000).

HSP have been associated to multidrug resistance in prostate cancers (Ciocca et al., 1993). Hsp60 and Hsp10 are constitutively expressed in most mammalian cells (Welch, 1992). Hsp60 functions as a protein chaperone and also is involved in protein folding. Cappello et al. showed that the expression of Hsp10 and Hsp60 were high in early prostate carcinogenesis (Cappello et al., 2003c). The overexpression of Hsp60 and its coregulator, Hsp10, in early prostate cancer, suggest that these two HSP may be important in prostate cancer development. Hsp90 is among the most abundant proteins of eukaryotic cells, comprising 1–2% of the cell's total proteins. Hsp90 interacts with several key proteins in promoting prostate cancer progression, including wild-type and mutated AR, HER2, ErbB2, Src, Abl, Raf and Akt. In prostate cancer, Hsp90 plays an important role in stabilization of the AR prior to ligand binding to Hsp90. This chaperone hetero-complex stabilizes the AR to be in a partially unfolded, high-affinity conformation, which is necessary for efficient response to androgens (Pratt et al., 2004). However, Hsp90-receptor hetero-complexes exist of the glucocorticoid receptor, progesterone receptor, mineralocorticoid receptor, and estrogen receptor (Prescott and Coetzee, 2006). The interaction of Hsp90 with the AR is one mechanism for the development of hormone resistance and represents the ligand-independent activation of the AR.

Plasma levels of Hsp70 have been shown to be higher in patients with localized untreated prostate cancer compared to controls (Abe et al., 2004). However the role of Hsp70 in prostate cancer is unclear until a recent study by Hurwitz and colleagues studied the biological significance of serum Hsp72 in patients undergoing radiation therapy (XRT) for prostate cancer with or without hormonal therapy (ADT). Circulating serum Hsp72 levels were shown to increase an average of 3.5-fold (median per patient 4.8-fold) with XRT but not with ADT, and that increases in IL-6 (3.3-fold), TNF-α (1.8-fold), $CD8^+$ CTL (2.1-fold) and NK cells (3.2-fold) also occurred. Using PC3 and DU145 human prostate tumor xenograph models in mice, these authors confirmed that XRT induces Hsp72 and Hsp27 release primarily from implanted tumors. Accompanying in vitro studies using supernatant recovered from irradiated human prostate cancer cells point to exosomes containing Hsp72 as a possible stimulator of pro-inflammatory cytokine production and costimulatory molecules expression in macrophages. Taken together these studies identify the released exosomes containing Hsp72 as playing a pivotal role in stimulating pro-inflammatory immune responses. These findings, if validated, may lead to new treatment paradigms for common human malignancies (Hurwitz et al., in preparation).

CONCLUSION

In conclusion, HSP are found within cells at basal levels and can be induced in response to various stressors. Aberrantly functioning cells, including cancer cells, often constitutively express high levels of HSP. Heat shock proteins have several important cellular functions: (1) protect other proteins against aggregation, (2) solubilize initial and loose protein aggregates, (3) assist in folding of nascent proteins or in refolding of damaged proteins, and (4) target severely damaged proteins to degradation and in case of excessive damage, sequester damaged proteins to larger aggregates. Overexpressions of HSP in cancers have beneficial for their survival and to develop resistance to varieties of anti-tumor drugs. Cancer cells acquire malignant capabilities by overexpressing HSP, which prevents apoptotic cell death. Amongst the molecular chaperones Hsp90 is critical for stabilizing many receptors, protein kinases and transcription factors. Hsp90 has over 200 client proteins, which are essential for signal transduction pathways that are ultimately converted in cancer. Hsp90 appears to play an important role in maintaining transformation by chaperoning an array of dysregulated, mutated proteins implicated in tumor growth and survival. Hsp90 is also a key anti-apoptotic regulator, conferring survival advantages to tumor cells. Recently, Hsp90 has been main target for therapeutic inhibitors, the most important Hsp90 inhibitors are geldanamycin and its less toxic analog, 17-allylamino-17-demethoxy-geldanamycin (17AAG) as well as radicicol. Inhibition of Hsp90 induces apoptosis of various tumor cells. Hsp90 inhibitors have been in Phase I and II clinical trials. Hsp70 and Hsp27 are crucial to the survival of a large proportion of human cancers and inactivation of or knockdown of these proteins leads to a spontaneous activation of programmed cell death. HSP have unique remarkable ability to interact with a wide range of proteins and peptides (antigenic peptides), a property that is shared by major histocompatibility complex molecules. HSP have been used in new immunotherapies of varieties of cancers. Increased Hsp60, Hsp70 and Hsp72 may lead to tumor cell sensitization for immune attacks by two mechanisms: tumor cells may express HSP on their surface, which leads to their enhanced recognition by the natural killer cells of the native immune system as well as a specific antitumor immunity may be induced by HSP-related antitumor vaccination. Several labs are currently involved in clinical trials based on HSP-peptide and NK cell immunotherapies for varieties of cancers. Early data show these agents to be efficacious.

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CHAPTER 8

HEAT SHOCK PROTEIN (HSP)-BASED IMMUNOTHERAPIES

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Abstract: Heat Shock Proteins (HSP) are a diverse group of proteins that as molecular chaperons bind to a variety of cell proteins in all cells. HSP also play a significant role in helping the immune system recognize diseased cells. During the past three decades, HSP are found to be a potent agent for tumor immunotherapy and studies towards anti-tumor vaccine development still continue today. HSP-based immunotherapies, which could augment antigen-specific immune responses, are the promising approaches for effective treatment and enduring cure. However, more studies on the role of HSP to induce innate and adaptive immune responses have resulted in new understanding of HSP in immunotherapy. Certain HSP are over expressed in tumor cells and aid tumor cells metastasis. Under this circumstance, HSP were chosen as targets for cancer treatments. Recent evidence further demonstrated that HSP possess immunoregulatory attributes. The high evolutionary conservancy of HSP and their overexpression during inflammation make them as important pathogen-related antigens as well as self antigens. Certain HSP (specifically Hsp60, Hsp65, Hsp70 and Hsp10) have been identified to be involved in the regulation of some autoimmune diseases. Now HSP becomes a double-sided sword. Therefore, HSP are also a tool for manipulating autoimmune inflammation and HSP for immunotherapy of autoimmune diseases are under discovery

Keywords: Cancer; chimeras; DNA vaccines; fusion proteins; heat shock proteins; immunotherapy

Abbreviations: AIA, adjuvant induced arthritis; APC, antigen presenting cells; BCG, Bacillus Calmette-Guérin; CTL, cytotoxic T lymphocytes; DC, dendritic cells; ERK, extracellular-signal regulated kinase; Grp, glucose regulated proteins; HER, human endothelial growth factor receptor; HLA, human leukocyte antigen system; HPV, human papilloma virus; HSF, heat shock factor; Hsp, heat shock proteins; *hsp*, heat

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shock protein gene; HSP, heat shock protein family; *HSP*, heat shock protein family gene; ICD, intracellular domain; IDDM, insulin-dependent diabetes mellitus; IFA, incomplete Freund's adjuvant; IL, interleukin; NHL, non-Hodgkin lymphoma; NO, nitric oxide; NOD, non-obese diabetic; RA, rheumatoid arthritis; RCC, renal cell carcinoma; RRP, recurrent respiratory papillomatosis; TAA, tumor-associated antigens; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha

INTRODUCTION

Heat shock proteins (HSP) are highly conserved proteins observed in all major cellular compartments and all organisms, from bacteria to mammalian, even plants (Lindquist and Craig, 1988). A large number of HSP have been identified, and are classified according to their respective molecular weights and their intracellular locations. One of the important functions of HSP is molecular chaperones, which facilitate in a wide variety of intracellular protein processes such as: the correct folding and translocation of newly synthesized proteins, the assembly and disassembly of protein complexes, and the refolding of misfolded proteins (Hartl, 1996; Wegele et al., 2004). In addition to the intracellular function as molecular chaperones, HSP have been suggested to be released in extracellular milieu through passive and active pathways (Asea, 2005; Campisi and Fleshner, 2003; Pockley, 2003), and play an important role in innate and adaptive immune responses (Calderwood et al., 2005; Srivastava, 2002). It has been reported that HSP-peptide complexes obtained from tumor tissue, reconstituted HSP-peptide complexes and even recombinant HSP-peptide fusion proteins can elicit strong antigen-specific CTL responses after immunization of mice. Certain HSP, including Hsp70 (Udono and Srivastava, 1993), Grp94 (Janetzki et al., 2000; Przepiorka and Srivastava, 1998), Hsp90 (Przepiorka and Srivastava, 1998), calreticulin (Basu and Srivastava, 1999), Hsp110 (Manjili et al., 2002) and Hsp170 (Wang et al., 2001), have been shown to act as adjuvants mediating the peptide cross-presentation, elevating CTL-specific responses and assisting the immune system to recognize tumor/diseased cells. Evidence indicates that the immunogenicity of HSP-peptide complexes has been exploited in the therapies of human cancers and infectious diseases (Przepiorka and Srivastava, 1998; Takakura et al., 2007; Wang et al., 2006). Clinical trials in melanoma and colorectal cancer have demonstrated that HSP-peptide complexes derived from autologous tumors can be used as vaccine to treat the same cancer (Belli et al., 2002; Srivastava, 2006).

Several specific characteristics of HSP have made them ideal adjuvants for immunotherapy for cancer treatments. First, as molecular chaperones, HSP are able to bind a large number of peptides and proteins. It was the peptides bound by HSP during the purification from tumor cells that elicited the immunity against cancer. The peptides bound by the HSP would act as the fingerprint of the tumor/cancer/diseased cells of origin, which immune system would recognize. However, without the assistance of HSP, the peptide itself would not do the trick (Takakura et al., 2007). Second, HSP can be efficiently taken up by specific receptors

on the surface of antigen presenting cells (APC). This is a crucial step for cross-presentation of the antigen peptides complexes with HSP. After taken up by APC, HSP-peptide complexes are processed in the endosome and the antigen peptide will be presented by MHC class I molecules, which primes $CD8^+$ CTL antigen-specific responses (Srivastava et al., 1994). Third, independent of chaperoned peptides, HSP themselves have been demonstrated to be involved in innate immune responses. HSP can activate maturation of DC, induce the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and tumor necrosis factor-α (TNF-α) and release of nitric oxide (NO), chemokines (RANETS, MCP-1) by macrophages and DC (Asea, 2005; Asea et al., 2000; Lehner et al., 2004; Wang et al., 2002). All of the specificities make HSP a potent agent for tumor immunotherapy and HSP-based immunotherapy is believed to be one of the most promising areas of developed cancer treatment.

With more studies of how HSP enhances the anti-tumor immunity carrying out, certain HSP are found to be over expressed on the surface of tumor cells and aid tumor cells metastasis. Under this circumstance, HSP were chosen as targets for cancer treatments. Moreover, recent evidence from animal models of autoimmune diseases has clearly demonstrated that HSP, particular Hsp60, have been associated with a number of human autoimmune diseases (Raska and Weigl, 2005). HSP have become a double-sided sword. In addition to stimulate immune responses, HSP are able to attenuate the immune responses under some circumstance. Studies towards important therapeutic implications for the future treatment of autoimmune diseases are under discovery.

HSP-PEPTIDE COMPLEXES (HSPPC) AS IMMUNOTHERAPY FOR CANCER

Srivastava et al. first reported that HSPPC-gp96 from tumor cells contain the HSP associated with cancer specific antigenic peptides (Srivastava and Das, 1984; Srivastava et al., 1986). Animals were immunized with HSP-peptide complexes (HSPPC) purified from autologous cancer tissue elicited strong tumor-specific immune responses and resulted in retarded progression of the primary cancer. The efficacy of HSPPC vaccination was later assessed in a series of animal models. Palladino et al. demonstrated that immunization with partially purified gp96 from a chemically induced murine fibrosarcoma (Meth A) protected mice from tumor growth after challenged with a large dose of Meth A cells (Palladino et al., 1987). The antigen-specificity is the most important characteristics of HSPPC immunogenicity. Tamura et al. examined the use of HSPPC complexes in the treatment of a variety of established cancers of spontaneous and experimental origin and observed the similar degree of antigen-specific anti-tumor immunity. The authors showed that the efficacy of autologous cancer-derived HSPPC-gp96 in immunotherapy of cancers (such as 3LL lung carcinoma, B16 melanoma, colon carcinoma and spindle cell carcinoma) (Tamura et al., 1997). In addition to gp96, a number of HSP, including

Hsp70, Hsp90, Hsp110 and calreticulin, later were identified to display similar antigen specific anti-tumor activity when purified from tumors (Takakura et al., 2007). Subjeck's group confirmed that prior vaccination with HSP110 or grp170 purified from methylcholanthrene-induced fibrosarcoma caused complete regression of the tumor in mice (Wang et al., 2001). Meanwhile, the same group checked the second tumor model and demonstrated that HSP110 or grp170 purified from Colon 26 tumors led to a significant growth inhibition of this tumor. In addition, HSP110 or grp170 immunization significantly extended the lifespan of Colon 26 tumor-bearing mice when applied after tumor transplantation. A tumor-specific cytotoxic T lymphocyte response developed in the mice immunized with tumor-derived HSP110 or grp170 (Wang et al., 2001). Faure et al. tested the immunogenicity of 2 epitotes from the sequence of the human HSP70, p391 and p393, which exhibit a high affinity for HLA-A*0201, expressed in many human tumor cells, including melanoma, breast cancer, colon and bladder carcinoma. They demonstrated that these HSP70 peptides were able to trigger a CTL response in vivo in HLA-A*0201-transgenic HHD mice and in vitro in HLA-A*0201+ healthy donors. p391- and p393-specific human and murine CTL recognized human tumor cells overexpressing HSP70 in a HLA-A*0201-restricted manner (Faure et al., 2004). In 2001, Sato et al. found that immunization BALB/c mice with HSP70 and GP96 purified from syngeneic leukemia cell line A20 elicited a specific response of potent CD8(+) T lymphocytes cytotoxic against A20 and prolonged mice survival after A20 inoculation (Sato et al., 2001). Later, Jimbo et al. also reported that immunization with A20 leukemia-derived Hsp70 induced the production of anti-A20-antibodies against leukemia-cell-specific peptides and play a crucial role in the eradication of leukemia cells in mice (Jimbo et al., 2008).

The success of tumor-derived HSPPC cancer vaccine in animal studies promoted its clinical exploration. Myeloma cell-derived HSPPC-96 was investigated as an effective immunotherapy for patients with multiple myeloma. Autologous, HLA-A0201 dendritic cells were pulsed with gp96 derived from HLA-A0201 human myeloma cell line U266 or primary myeloma cells. Specific CTL cell lines were obtained after repeatedly stimulating T cells with these pg96-pulsed dendritic cells. These specific CTL cells were able to effectively lyse myeloma cells but not normal blood cells. This also suggested that HSP from allogeneic tumor cells may be used as vaccines to immunize patients (Qian et al., 2005)

Vaccination with HSPPC-gp96 from autologous liver metastases of colorectal carcinoma in patients with colon cancer underwent radical resection of liver metastases was also tested. It demonstrated that the HSPPC-gp96 vaccination elicited a significant increase in $CD8^+$ T-cell response against colon cancer (Mazzaferro et al., 2003). Phase II trial was also to investigate the safety and efficacy of autologous HSPPC-96 vaccines prepared from tumor specimens of patients with newly diagnosed or previously treated indolent non-Hodgkin lymphoma (NHL), but it had limited efficacy in inducing responses in patients with active diseases (Oki et al., 2007). Phase I/II clinical studies using tumor-derived HSPPC-gp96 as vaccines are ongoing for melanoma, colorectal carcinoma and kidney cancer (Parmiani et al.,

2004; Takakura et al., 2007; Wang et al., 2006). In most of these clinical trials, the results indicated that HSPPC-gp96 vaccination is safe and elicited potent antigen-specific CTL responses against the autologous tumors. One of the most intensive clinical trials has been conducted on HSPPC-gp96 (Oncophage, vitespen; Antigenics Inc) for the treatment of metastatic renal cell carcinoma (RCC) (Gordon and Clark, 2004). Metastatic RCC is a disease with a serious prognosis. The 1-year survival rate declines to approximately 60% as metastasis is diagnosed. The well-known but occasional occurrence of spontaneous or nephrectomy induced regression of metastasis in patients with RCC and the existence of tumor-reactive and tumor infiltrating CTL imply that RCC is an immunogenic tumor (Marcus et al., 1993). Investigations into the role of heat shock proteins (HSP) in immune response have progressed well into a third decade, and use of HSPPC-gp96 for the treatment of renal cell carcinoma (RCC) in the adjuvant setting already showed good efficacy (Aalamian et al., 2006). The clinical trials were carried out in patients underwent nephrectomy and vaccine was prepared from the primary tumor. For these patients, no proved treatment exists beyond nephrectomy. In the first study, this HSPPC-gp96 vaccine was manufactured for clinical administration for 38 patients. Of evaluable patients who received one complete dose, 32% (12 out of 37) had a response to the vaccine. In the second study, in 62 evaluable patients, a 15% (9 out of 61) response rate was obtained (Aalamian et al., 2006). With support of these data, more random phase III trials have been conducted. Analysis of 604 patients with metastasis RCC underwent nephrectomy demonstrated that Oncophage (vitespen; formerly HSPPC-96; Antigenics Inc., New York, NY, USA) is easy to administer, appears well tolerated, shows antitumor activity and significantly improves the recurrence-free survival rated in patients with metastatic RCC (Antigenics, 2006). Last year, Oncophage was approved in Russia for the treatment of intermediate-risk kidney cancer. Findings from the largest, randomized kidney cancer trail ever completed in the adjuvant treatment setting showed that patients receiveing Oncophage in the imtermediated-risk population demonstrated approximately 45% improved survival rate. This registration of Oncophage in Russia represents an important treatment advancement for patients with intermediate-risk kidney cancer (Antigenics, 2008).

Although the tumor-derived HSPPC is a very promising immunotherapy for cancer treatments, the sufficient amount of specimen for purification of HSPPC is always a problem. Therefore, new approaches, such as reconstituted HSP-peptide complexes and HSP-peptide fusion proteins, are developed to overcome the limitations of tumor-derived HSPPC. Manjili et al. took advantage of more binding efficiency of large HSP to reconstitute Hsp110 and intracellular domain (ICD) of human HER-2/neu complexes. This non-covalent Hsp110-ICD complexes, which is a fourfold more efficient in binding to protein substrates compared to Hsp70 had elicited effective IFN-γ-producing T cells against spontaneous mammary tumors in FVB-neu transgenic mice (Manjili et al., 2002; Manjili et al., 2003). Later, the same group reconstituted Hsp110-gp100 chaperone complex and demonstrated that immunization with the hsp110-gp100 complex protected mice against subsequent challenge with human gp100-transduced B16 melanoma (Wang et al., 2003).

Compared to reconstituted HSP-peptide complexes, recombinant HSP-peptide fusion proteins have more advantages. The antigenic peptide can be as small as several amino acids and the peptide is covalently linked to HSP to ensure each HSP molecule has its "dangerous signal". In 1997, Suzue et al. first developed recombinant mycobacterial Hsp70 and a large fragment of ovalbumin fusion protein. The authors demonstrated that the recombinant soluble fusion protein, in the absence of adjuvants, stimulated H-2b mice to produce ovalbumin-specific $CD8^+$ CTL. The CTL recognized an immunodominant ovalbumin-derived peptide, SIINFEKL, known to be a naturally processed peptide derived from ovalbumin expressed in mouse cells (Suzue et al., 1997). Later the immunogenicity of different recombinant HSP-peptide fusion proteins was carried out. An HSP fusion protein called HSP-E7 composed of *Mycobacterium bovis* BCG Hsp65 linked to E7 protein of human papillomaviruses type 16 (HPV16) was developed and it can eradicate the outgrowth of established TC-1 tumors (a HPV16 E7-expressing tumor cell line) in mice. More studies about HSP-E7 showed that HSP-E7 can primes potent E7-specific $CD8^+$ T cells with cytolytic and cytokine secretion activities (Chu et al., 2000a, b). Even fusion protein containing truncated Hsp70 (N-terminal or C-terminal domain) linked to HPV16-E7 are capable of inducing potent antigen-specific CTL activity in animal models. E7 protein is involved in carcinogenesis of anal and cervical tumors, and represents a tumor antigen that may be specifically targeted by lymphocytes (Liu et al., 2008). Therefore, HSP-E7 as a single-agent therapy was advanced into multiple phase II clinical trials with positive results, including trials in cervical dysplasia and recurrent respiratory papillomatosis (RRP). Now HSP-E7 as a potential therapy for conditions associated with HPV infection is currently undergoing phase III clinical trials (Maciag and Paterson, 2005).

HSP-BASED DNA VACCINES

Plasmid DNA has been shown to be capable of inducing both humoral and cellular immunity against the expressed proteins. Due to their stability and simplicity of delivery, DNA vaccines have become an attractive immunotherapy against various diseases including cancer, allergy and chronic diseases (Liu and Ulmer, 2005). Therefore, HSP-based DNA vaccination has also been carried out by several research groups. Chen et al. made use of human papillomavirus type 16 E7 (HPV-E7) as a model antigen and evaluated the effect of linkage to *Mycobacterium tuberculosis* Hsp70 on the potency of antigen-specific immunity generated by naked DNA vaccines (Chen et al., 2000). These authors demonstrated that vaccines containing E7-Hsp70 fusion genes increased the frequency of E7-specific $CD8^+$ T cells by at least 30-fold relative to vaccines containing the wild-type E7 gene. More importantly, the E7-Hsp70 DNA vaccine also enhanced significant potency against established E7-expressing tumors. Moreover, immunological and antitumor effects of E7-Hsp70 DNA vaccines were completely CD4-independent. The DNA vaccine exclusively targeted $CD8^+$ T cells. Hauser et al. described a novel DNA vaccination strategy

based on the targeting of a modified tumor-associated antigen, the human papilloma virus type 16 E7 protein (HPV-E7), to DC by a HSP to enhance antigen presentation and immune responses. Specifically, a chimerical HPV-E7 and Hsp70 fusion gene preceded with a leader sequence was constructed. When mice were immunized with this construct, the DNA is taken up by various types of cells, which then produce and secrete an HPV-E7-Hsp70 fusion protein that is targeted to DC by the Hsp70 portion of the chimerical molecule for antigen presentation. The authors demonstrated that DNA vaccination with this secretory HPV-E7-Hsp70 construct strongly enhanced an antigen-specific $CD8^+$ T-cell response as well as a specific B-cell response in mice. Furthermore, this immunization approach not only protected mice against lethal challenge with an HPV E7-expressing tumor line (TC-1), but also showed a therapeutic effect against established tumors (Hauser et al., 2004). Later Wang's group explored the efficacy of mouse Hsp70 and Hsp110-based DNA vaccination against HPV-E7 tumor. These authors obtained similar results demonstrating that E7-Hsp70 DNA vaccine not only elicited an E7-specific CTL response, but also protected mice against challenge with E7 expressing tumors, and that the peptide-binding region, not the ATPase domain of Hsp70, is required for the vaccine activity of the E7-Hsp70 DNA (Li et al., 2006).

Ren et al. (2004) developed an in vivo HSP-suicide gene tumor vaccine by generating a recombinant replication-defective adenovirus (Ad-HT) that coexpresses Hsp70 and a herpes simplex virus thymidine kinase suicide gene. The combination of Hsp70 overexpression in situ and tumor killing by thymidine kinase/ganciclovir treatment, but neither strategy alone, provoked potent systemic antitumor activities after intratumor injection of Ad-HT. These findings have broad relevance to the use of the in vivo HSP/suicide gene tumor vaccine in therapy for human solid tumors.

Recent report by Schirmbeck et al. (2006) showed a specific DNA vaccine encoding HSP-capturing, chimeric peptides containing antigenic determinants of the tumor-associated antigens (TAA) gp70 (an envelope protein of endogenous retrovirus). An approximately 200 residue gp70 fragment or its L(d)-binding antigenic AH1 peptide cloned in-frame behind an HSP-capturing (cT(272)) or non-capturing (T(60)) N-terminal large SV40 tumor Ag sequence was expressed as either HSP-binding or HSP-non-binding chimeric antigens. Only HSP-capturing, chimeric fusion proteins were expressed efficiently in transfected cell lines and primed TAA-specific $CD8^+$ T cell immunity. These results indicate that a vaccination strategy based on delivering antigenic, HSP-associated TAA fragments can thus prime protective $CD8^+$ T cell immunity even if these TAA are of low intrinsic immunogenicity.

HSP AS TARGETS FOR CANCER TREATMENTS

HSP-peptide complexes (such as gp96 and HSP70) either derived from autologous tumor or reconstructed in vitro can initiate tumor-specific protective CTL immunity and their application in immunotherapy for cancer treatments have been

rigorously carried out. However, the reports about the tumor-specific immunogenicity of HSP90-peptide complexes are very limited (Kurotaki et al., 2007; Udono and Srivastava, 1994). More research work on HSP90 has been identified HSP90 as a critical modular of numerous cellular process, which are usually deregulated in tumor cells. HSP90 is constitutively expressed at higher level in tumor cells. HSP90 plays an important role for tumor growth and survival and facilitate malignant progression of tumor (Cullinan and Whitesell, 2006; Kamal et al., 2003). HSP90 has emerged as an exciting molecular target for cancer therapy. The client proteins HSP90 chaperoned include the serine/threonine kinase Raf-1, oncogenic tyrosine kinase v-Src, mutant p53, ERBB2 and mutant oncogene Bcr/Ab1 (Sharp and Workman, 2006; Tsutsumi and Neckers, 2007). HSP90 inhibitors like geldanamycin (GA), first reported by Whitesell et al. inhibited the association of the chaperone with the client proteins and eventually led to degradation of HSP90 client proteins (Whitesell et al., 1994). However GA itself proved to be too hepatotoxic for clinical use. The first-in-class HSP90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), is a better tolerated derivative of GA. It has shown promising anti-tumor immunity and currently in phase II clinical trials in various tumor types including melanoma and breast cancer (Sharp and Workman, 2006).

Both 17-AAG and GA bind to a conserved structure in HSP90 family and interrupt the intrinsic ATPase activity of HSP90. The toxicity of 17-AAG has been mild. Several preclinical studies have shown that 17-AAG may enhance the efficacy of a variety of chemotherapeutic agents. Pharmacodynamic endpoints, such as induction of HSP70 and downregulation of C-RAF and CDK4 in peripheral blood mononuclear cells and tumor biopsies from treated patients, provided evidence of HSP90 inhibition at well-tolerated doses. The completed phase I trials of 17-AAD have shown favorable results. Goetz et al. determined the maximum-tolerated dose (MTD) of 17-AAG when infused on days 1, 8, and 15 of a 28-day cycle in advanced solid tumor patients is 308 mg/m^2. 17-AAG resulted in good pharmacokinetic exposures and induced HSP70 in PBMCs, indicating that Hsp90 has been affected (Goetz et al., 2005). Banerji et al. also demonstrated 17-AAG exhibits a tolerable toxicity profile with therapeutic plasma concentrations and target inhibition for 24 h after treatment (Banerji et al., 2005). 17-AAG has been tested for treatment of melanoma because it can block both the MAPK and ART signaling pathways. Solit et al. conducted a phase II trial using 17-AAG in melanoma patients. In fifteen evaluable patients, nine had BRAF mutations and six were wild-type. No objective responses were observed. Western blot analysis of tumor biopsies showed an increase in HSP70 and a decrease in cyclin D1 expression in the posttreatment biopsies but no significant effect on RAF kinases or phospho-extracellular signal-regulated kinase expression (Solit et al., 2008).

In addition to HSP90, HSP25, a murine homolog of human HSP27, has been reported to regulate tumor growth and metastasis. Bausero et al. showed that 4T1 mammary adenocarcinoma cells with high-expressed HSP25 grew significantly faster than those with low-expressed HSP25 (Bausero et al., 2004). Sliencing the *hsp*25 gene eliminates migration capability of the metastatic 4T1 cells (Bausero

et al., 2005). Later, Mori-Iwamoto et al. proved HSP27 as a biomarker for resistance of pancreatic cancer cells to gemcitabine using proteomics. Pancreatic cancer remains a devastating disease and >96% of patients with pancreatic cancer do not survive for more than 5 years. Gemcitabine (2′-deoxy-2′-difluoro-deoxycytidine: Gemzar) appears to be the only clinically effective drug for pancreatic cancer, but it has little impact on outcome. The authors demonstrated that knocked down HSP27 in KLM1-R can restore the sensitivity to gemcitabine. In addition, increased HSP27 expression in tumor specimens was related to higher resistibility to gemcitabine in patients of pancreatic cancer. Proteomic analysis of gemcitabine-sensitive cells (KLM1) and resistant pancreatic cells (KLM1-R) was performed to identify target proteins of the gemcitabine (Mori-Iwamoto et al., 2007). Aloy et al. also demonstrated the protective role of HSP27 against radiation-induced apoptosis. Attenuation of HSP27 expression was accomplished by antisense or RNAi (interfering RNA) strategies in SQ20B resulted in increased apoptosis, decreased glutathione basal level, and clonogenic cell death (Aloy et al., 2008). All the results indicate anti-HSP27 gene therapy offers a potential adjuvant to radiation-based therapy of resistant tumors.

HSP AS IMMUNOTHERAPY FOR AUTOIMMUNE DISEASES

HSP can augment antigen-specific immune responses and several types of HSP-based immunotherapy for cancer treatment have been rigorously explored. However, recent evidence demonstrated that HSP possess immunoregulatory attributes. With the contribution of HSP, the immune system is aware about the presence of the foreign pathogens. The high evolutionary conservancy of the bacterial HSP and their overexpression during inflammation make them as important pathogen-related antigens as well as self antigens. Bacterial HSP are immunodominant antigens that are cross-reactive with their mammalian counterparts. Therefore, HSP in microorganisms that commonly infect humans may be triggers of humoral and cellular autoimmune responses and consequent overt autoimmune disease expression. On the contrary of sending "danger signal" to immune system, this time the HSP themselves as "danger signal" stimulate immune responses. The findings have clearly demonstrated immune responses to HSP are associated with both induced and spontaneous autoimmune diseases. Certain HSP (specifically Hsp60, Hsp65, Hsp70 and Hsp10) have been identified to be involved in the regulation of some autoimmune diseases such as rheumatoid arthritis (RA), atherosclerosis and insulin-dependent diabetes mellitus (IDDM) (Raska and Weigl, 2005). Therefore, HSP have the capability of modulating autoimmune processes and are promising candidates for immunotherapy of autoimmune diseases.

Insulin-dependent diabetes mellitus is caused by autoimmune destruction of the insulin-producing beta cells of the pancreas (Rossini et al., 1985). Elias et al. first reported that a beta-cell target antigen in non-obese diabetic (NOD/Lt) mice is a molecule cross-reactive with the 65-kDa heat shock protein (Hsp65) of

Mycobacterium tuberculosis and the onset of beta-cell destruction is associated with the spontaneous development of anti-Hsp65 T lymphocytes (Elias et al., 1990). The ability of clones of anti-Hsp65 T cells to cause insulitis and hyperglycemia in young NOD/Lt mice further demonstrated that importance of Hsp65 in the pathogenesis of insulin-dependent diabetes. Moreover, Hsp65 antigen could be used either to induce diabetes or to vaccinate against diabetes, depending on the form of its administration to pre-diabetic NOD/Lt mice. The immunogenic Hsp65 administered in incomplete Freund's adjuvant (IFA) induced anti-Hsp65 T lymphocytes and a self-limited form of IDDM. However, vaccination of Hsp65 in PBS protected the mice against anti-Hsp65 T lymphocytes response to Hsp65 in IFA, and effectively aborted the chromic spontaneous disease. Later, Saruta group also demonstrated that immune response to Hsp65 correlates with insulitis in NOD mice (Shimada et al., 1996).

Rheumatoid arthritis (RA) is an inflammatory disease that primarily involves the joints. By now, more and more evidence has been obtained that preimmunization with different microbial HSP protect from subsequent arthritis induction (Puga Yung et al., 2003). The microbial HSP include Hsp60 (Prakken et al., 2002), Hsp70 (Wendling et al., 2000) and Hsp10 (Ragno et al., 1996). Recent progress has shown promising results using HSP peptides to modulate the adjuvant induced arthritis (AIA), a RA rat model. Nasal administration of an altered peptide ligand of Hsp60 (180–188 T cell epitope) to rat before and after induction of arthritis highly effective protection against AIA through generation of regulatory cells that produce IL-4, TGF-β and IL-10, whereas the induced tolerance is driven mainly by production of IL-10 (Prakken et al., 2002). Wendling et al. studied the nature of the arthritis suppressive capacity of a distinct, antigenically unrelated protein, M. tuberculosis Hsp70 and found nasal administration of HspP70 peptide triggers self-HSP cross-reactive T cells with the potential to down-regulate arthritis via IL-10 (Wendling et al., 2000). Rango et al. first reported the immunomodulatory activity of mycobacterial Hsp10 in experimental arthritis (Ragno et al., 1996). These results demonstrate that an aqueous solution of a mycobacterial Hsp10 delayed the onset and severity of adjuvant-induced arthritis in rodents when administered after disease induction but before joint involvement occurred.

Atherosclerosis is a chronic inflammatory disease. Many studies and observations suggest that it could be caused by an immune reaction against autoantigens at the endothelial level, the most relevant of which are oxidized LDL. More recent research has demonstrated that HSP play a primary antigenic role in the autoimmune pathogenesis of atherosclerosis (Blasi, 2008; Rigano et al., 2007). Especially Hsp60-specific T cells contribute to the development of the immune responses in atherosclerosis. High level of Hsp60-specific antibodies was detected in serum of patients with atherosclerosis (Xu et al., 1994). Benagiano et al. reported that atherosclerotic plaques harbor in vivo-activated $CD4^+$ T cells that recognize the human Hsp60 (Benagiano et al., 2005). The activated $CD4^+$ T cells characterized the submolecular specificity of such Hsp60-specific plaque-derived T cells and identified both the self- and cross-reactive epitopes of that autoantigen. The results indicated that arterial endothelial cells, undergoing classical atherosclerosis risk factors and

conditioned by Th type 1 cytokines, express self Hsp60, which becomes target for both autoreactive T cells and cross-reactive T cells to microbial Hsp60 via a mechanism of molecular mimicry. van Puijvelde et al. administered Hsp60 and the peptide Hsp60 (253–268) orally to $LDLr^{-/-}$ mice before induction of atherosclerosis and resulted in a significant 80% reduction in plaque size in the carotid arteries and in a 27% reduction in plaque size at the aortic root. The data indicated that oral tolerance induction to Hsp60 and a small Hsp60-peptide leads to an increase in the number of $CD4^+/CD25^+/Foxp3^+$ regulatory T cells, resulting in a decrease in plaque size as a consequence of increased production of IL-10 and TGF-β (van Puijvelde et al., 2007). All the beneficial results may provide new HSP-based immunotreatment for atherosclerosis.

CONCLUSION

HSP are now recognized to interact with the immune system in different ways. The correct use of the regulation of HSP to the immune responses in the formulation of new approaches to immunotherapy of cancers, infectious diseases and autoimmune diseases has now attracted more attention of more and more researchers. However, the mechanism of HSP in regulation of immune responses remains unclear. Many new discoveries and applications can be expected.

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CHAPTER 9

HEAT SHOCK PROTEINS AND FERTILITY

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Abstract: Rapid cell growth and differentiation, hallmarks of gametogenesis, fertilization, and early embryo development, require the participation of heat shock proteins. Spermatozoa formation and maturation in mammals occurs outside of the body cavity since this process is optimal at a lowered temperature. Heat shock transcription factors (HSF) have specialized functions in spermatogenesis. HSF1 becomes activated in the testes in response to elevated temperature and protects spermatogonia viability. Conversely, the same HSF1 induces apoptosis of spermatocytes that are produced under adverse conditions, insuring that defective spermatozoa are not maintained. A novel HSF, HSFY, is coded by a gene on the Y chromosome; its role during spermatogenesis remains undefined. A testes-specific heat shock protein, hspA2, is essential for male, but not female, germ cell meiosis. Spermatozoa acquire the capacity for fertilization following their deposition in the female genital tract. Two heat shock proteins, HSPD1 and HSP90B1, become exposed on the sperm head, undergo phosphorylation and may direct formation of a complex that binds to a glycoprotein on the surface of the zona pellucida. The constitutive form of the 70 kDa heat shock protein, as well as the 60 kDa heat shock protein (HSP60), appear to function during oogenesis. Preimplantation stage embryo development requires heat shock protein participation. Monoclonal antibodies to heat shock proteins block the in vitro development of mouse and bovine zygotes. The presence of antibodies to heat shock proteins is associated with a failure to become pregnant in women undergoing in vitro fertilization (IVF). *Chlamydia trachomatis* infection of Fallopian tube epithelial cells can lead to development of immunity to conserved regions of the microbial HSP60 that are also expressed on the homologous human HSP60. This interferes with subsequent attempts at conception, either naturally or by IVF. The 70 kDa heat shock protein is also present in amniotic fluid where it may function in the modulation of immune responses

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Abbreviations: HSF, heat shock transcription factors; BV, bacterial vaginosis; IVF, in vitro fertilization; SC, synaptonemal complex; TNF-α, tumor necrosis factor-alpha

INTRODUCTION

The processes of fertilization, cell growth and differentiation, and the need of the developing semi-allogeneic fetus to modulate maternal immune system responses, requires the precise functioning of developmental events in a clearly defined sequence. Heat shock proteins play a universal role as molecular chaperones throughout evolution, fostering the movement, correct folding and assembly of polypeptides as well as marking defective proteins for removal. The amino acid sequence of heat shock proteins is highly conserved and they are essential for survival in all organisms from bacteria to plants to mammals (Hunt and Morimoto, 1985). It is intuitive, therefore, that heat shock proteins would be intimately involved in reproductive processes. While the precise functions of individual heat shock proteins in fertility remain incompletely elucidated, research in this area of investigation is becoming increasingly prevalent. What is already apparent is that heat shock proteins produced by the host at different developmental stages as well as heat shock protein expression by microorganisms present in the female reproductive tract can affect fertility-related events. Immune responses to mammalian and microbial heat shock proteins also appear to either negatively or positively influence reproductive outcome.

Any investigation of the possible involvement of heat shock proteins in fertility must encompass the fields of microbiology and immunology. Individual variations in the endogenous microbial flora, or the presence of a microbial infection in the male or female reproductive tract, can directly affect the magnitude of human and microbial heat shock protein expression. Differences in the qualitative and quantitative production of heat shock proteins will also influence the extent of heat shock protein-related immunity and its subsequent effect on developmental processes.

This review will focus on the identification of heat shock proteins and their potential roles in gametogenesis, fertilization, and early embryonic development. Conditions fostering development of immunity to heat shock proteins and their effect on these reproductive processes will also be addressed.

SPERMATOGENESIS

In most mammalian species the testes are present in a scrotal sac that is located outside of the body cavity. The process of spermatogenesis, therefore, occurs at a temperature that is significantly lower than the internal body temperature. Even small elevations in scrotal temperature result in an impairment of sperm production and a reduction in fertility. Therefore, spermatogenesis is one of the biological

processes most sensitive to heat stress. Spermatogenesis consists of an ordered developmental sequence whereby diploid spermatogonia stem cells are transformed first into primary diploid spermatocytes and then into secondary haploid spermatocytes. These spermatocytes then rapidly divide to form spermatids. The spermatids do not undergo further cell division but go through a complex process of differentiation into mature spermatozoa while traversing through the male genital tract. Spermatozoa only acquire the ability to fertilize an ovum after they undergo a further change, called capacitation, following their ejaculation into the female genital tract.

Unique species of heat shock proteins and heat shock transcription factors with distinctive functions have been identified at different stages of male germ cell development. A testis-specific heat shock protein, hspA2, formerly called hsp70-2, is a variant member of the 70 kDa heat shock protein family. It is expressed at high levels in spermatocytes and appears to have an essential role during the meiotic phase of spermatogenesis. Homologous hspA2-related genes have been identified in germ cells from mammals, birds, amphibians and fish (Eddy, 1999). Male mice with a deletion in hspA2 are infertile. There is arrested germ cell development at the meiotic phase and the spermatocytes undergo massive apoptosis (Dix et al., 1996a). Similarly, some men with abnormal spermatogenesis were found to be deficient in hspA2 production (Son et al., 2000). Interestingly, female hspA2 knockout mice remain fertile (Dix et al., 1996b), indicating that oocyte meiosis is hspA2-independent. HspA2 is a component of the synaptonemal complex (SC), a chromosome-bound structure formed during meiosis (Dix et al., 1996a). It is absent from the SC formed during oocyte meiosis (Allen et al., 1996). In hspA2 knockout mice the SC is present but the chromosomes do not undergo meiosis (Dix et al., 1996a).

HspA2 also appears to be involved in formation of a Cdc2-cyclin B1 complex (Zhu et al., 1997). The acquisition of protein kinase activity following formation of this complex is required for spermatocytes to be able to undergo meiosis. Hsp70A2 binds to the Cdc2 component and facilitates a change in conformation resulting in its ability to form a heterodimer with cyclin B1.

A third putative role for hspA2 in nuclear reorganization has recently been proposed (Govin et al., 2006). Based on studies in mice, it appears that hspA2 also functions in the post-meiotic phase of spermatogenesis as a chaperone for the transition proteins TP1 and TP2 that replace histones on the nuclear DNA of spermatids.

The production of heat shock proteins is regulated at the level of transcription by heat shock transcription factors (HSFs). Similar to the role heat shock proteins, HSFs also appear to have unique roles during spermatogenesis. In somatic cells heat shock protein production in response to biological stressors is initiated by the conversion of inactive HSF1 into an active form capable of initiating gene transcription. However, during spermatogenesis activation of HSF1 does not result in induction of heat shock protein synthesis. Instead, it induces apoptosis of spermatocytes (Izu et al., 2004). It is proposed that spermatocytes exposed to unfavorable environmental conditions, and which, therefore, may be defective, are eliminated by HSF1 that

has been activated by the same stressful environment. This mechanism has obvious species survival value. Surprisingly, the ability of HSF1 to perform the opposite function and aid survival of undifferentiated spermatogonia following exposure to elevated temperatures has also been proposed (Izu et al., 2004).

A novel HSF, HSFY, coded by a gene on the Y chromosome, has also been suggested to have a role in spermatogenesis (Shinka et al., 2004). Although its specific function remains unclear, HSFY is expressed in spermatogenic cells and is transferred from the cytoplasm to the nucleus in a stage-dependent manner. In addition, two azospermic men were found to lack the HSFY gene. Sequences similar to the mouse HSFY gene have been identified in dogs, cows and chickens (Kinoshita et al., 2006).

FERTILIZATION

Fertilization involves the fusion of a single spermatozoa with an oocyte. Before this can be accomplished ejaculated sperm undergo a series of maturation steps in the female genital tract resulting in the exposure of components on the sperm head that are capable of recognizing and binding to a specific glycoprotein, ZP3, on the extracellular matrix surrounding the oocyte (zona pellucida). This maturation process, called capacitation, involves the phosphorylation of tyrosine residues on proteins present in the rostral region of the sperm head (Asquith et al., 2004). Two of these phosphorylated proteins are the heat shock proteins, HSPD1 (formerly called HSP60) and HSP90B1 (formerly called endoplasmin). A third heat shock protein, HSPE1 (formerly called HSP10), that becomes associated with HSPD1 on the sperm surface has also been identified (Walsh et al., 2008). It is hypothesized that these heat shock proteins, following their activation, direct the formation on the sperm head of a complex capable of binding to the zona pellucida.

Ejaculated semen also induces the production of messenger RNA for the 70 kDa heat shock protein (hsp70) in the human endocervix (Jeremias et al, 1997). Whether this is a protective physiological response of endocervical cells to exposure to male seminal constituents and/or a mechanism to protect sperm surface components from degradative enzymes in the female genital tract remains to be determined.

OOGENESIS

The appearance of heat shock proteins during oogenesis has been examined to a far lesser extent than during spermatogenesis (reviewed in Neuer et al., 2000). Expression of the constitutive form of the 70 kDa heat shock protein (HSC70) is high in murine pre-ovulatory oocytes and becomes undetectable in ovulated oocytes (Curci et al., 1991). The 60 kDa heat shock protein (hsp60) has been identified in follicular fluid obtained during oocyte retrieval from women undergoing a cycle of in vitro fertilization (Neuer et al., 1997). Recent studies in *Drosophila melanogaster* have identified a member of the hsp60 family, HSP60C, as being essential for

multiple steps in fruit fly oogenesis, principally related to formation of cytoskeleton structures (Sarkar and Lakhotia, 2008). Interestingly, the presence of antibodies to hsp60 in human follicular fluid has been correlated with a reduced pregnancy rate after in vitro fertilization (Cortinas et al., 2004; Jakus et al., 2008). Although other interpretations are possible, their findings are consistent with a requirement for hsp60 in the production of functional oocytes.

FEMALE REPRODUCTIVE TRACT INFECTION AND FERTILITY

The vaginal flora of reproductive age women is dominated by lactobacillus species, principally *L. crispatus, L. gasseri* and *L. jensenii*. This contributes to an acidic vaginal pH ($\leq$4.5) and prevents the overgrowth of other, potentially pathogenic, microorganisms. For reasons still not understood, in some women levels of vaginal lactobacilli become greatly reduced or disappear altogether and are replaced by several species of anaerobic bacteria, *Gardnerella vaginalis* and *Mycoplasma hominis*. This alteration, called bacterial vaginosis (BV), is associated with an elevation in vaginal pH and a greatly increased vaginal total bacterial concentration (Bartlett et al., 1977). BV has been associated with anovulation (Wilson et al., 2002), unexplained infertility (Spandorfer et al., 2001), occluded Fallopian tubes (Liversedge et al., 1999) and risk of first trimester miscarriage after in vitro fertilization (Ralph et al., 1999). BV also induces elevated vaginal levels of the 70 kDa heat shock protein (hsp70) (Giraldo et al., 1999a). Concomitant with hsp70 production are increases in vaginal concentrations of the anti-inflammatory cytokines, interleukin (IL)-10 (Giraldo et al., 1999b) and IL-1 receptor antagonist (Genc et al., 2005). Hsp70 gene transcription is known to block the transcription of genes coding for pro-inflammatory cytokines (Cahill et al., 1996). Thus, this induction of hsp70 and inhibitors of inflammation, coupled with the hsp70-directed blockage of pro-inflammatory mediator production, strongly suggests that the heat shock response is activated by an altered vaginal flora. This activity may function to limit local inflammation and subsequent damage to the vaginal epithelia. Epithelial integrity is essential to maintenance of the protective physical barrier at this site. Similarly, it has been hypothesized that endogenous intestinal bacteria (Kojima et al., 2003) or their metabolic products (Arvans et al., 2005) induce hsp70 production and in so doing enhance the viability of intestinal epithelial cells in the presence of potentially pathogenic microorganisms. A prolonged strong pro-inflammatory immune response also promotes development of antisperm immunity in women (Witkin and David, 1988).

PREIMPLANTATION EMBRYO DEVELOPMENT

The newly formed embryo initiates gene transcription at the 2 cell stage. In mouse embryos the constitutive form of hsp70 (HSC70) is among the first proteins evident at this earliest developmental stage (Morange et al., 1984). The inducible

hsp70 is first identified in blastocysts when differentiation into the inner and outer cell mass occurs (Wittig et al., 1983). The addition of monoclonal antibody to the inducible hsp70 to two cell mouse embryos cultured in vitro led to a potent inhibition of day 5 blastocyst development (Neuer et al., 1998) and induction of apoptosis (Neuer et al., 2000). Similarly, in in vitro grown bovine embryos, addition of anti-hsp70 significantly inhibited blastocyst development and induced apoptosis (Matwee et al., 2001). Addition of monoclonal antibodies to hsp60 and hsp90 also inhibited mouse embryo development in vitro, but at different stages from that of anti-hsp70. Anti-hsp60 had an early detrimental effect on day 3 blastocysts while anti-hsp90 appeared to only influence embryo development at day 7 (Neuer et al., 1998). Thus, it appears that expression of multiple heat shock proteins is required for optimal early embryo development. A limitation of these studies, however, is that the development of embryos under in vitro conditions is not identical to the in vivo milieu. It is possible that stress to the embryonic cells due to their development in a synthetic medium led to an altered or increased heat shock protein expression that might not parallel the natural situation and, furthermore, may have resulted in an atypical response following exposure to anti-heat shock protein monoclonal antibodies.

CHLAMYDIA TRACHOMATIS GENITAL TRACT INFECTION

Arguing against the potential artifact mentioned above are studies that demonstrate the detrimental effects of immunity to the *C. trachomatis* 60 kDa heat shock protein (chsp60) on human fertility. *C. trachomatis* is an obligate intracellular bacterium, the major sexually transmitted bacterial pathogen in developed countries and a leading cause of infertility or ectopic pregnancy due to Fallopian tube blockage (Paavonen 1992). Most female genital tract chlamydial infections are asymptomatic and, therefore, tend to become chronic. Many women only learn that they have been infected when they seek treatment for infertility and are found to have occluded Fallopian tubes and are positive for antibodies to *C. trachomatis*.

A chlamydial infection of tubal epithelial cells and release of infectious chlamydial elementary bodies into the extracellular environment elicits the host's immune response. Interferon-γ production activates phagocytic cells to destroy the extracellular form of the organism. However, interferon also interrupts the intracellular chlamydial growth cycle and induce the organism to enter a dormant, but viable, state. In this persistent form, *C. trachomatis* ceases to produce most of its components. However, synthesis of chsp60 is increased, presumably to aid bacterial survival under these adverse conditions. The chsp60 is also released into the extracellular space. This sequence of events is reviewed in Witkin (2002). The prolonged release of chsp60 and its continual activation of the maternal immune system eventually results in damage to the Fallopian tube epithelium, scar formation and occlusion. In addition, exposure to chsp60 over a long period of time increases the likelihood of formation of humoral (Witkin et al., 1998; Domeika et al., 1998) and cell-mediated (Witkin et al., 1993, 1994a) immunity to epitopes that are also

expressed in the homologous human hsp60. Women with blocked Fallopian tubes now seek to become pregnant by undergoing in vitro fertilization (IVF). Those women with chlamydial-related tubal infertility who are positive for antibodies to chsp60 or the human hsp60 have a reduced IVF success rate as compared to women who are negative for these antibodies (Witkin et al., 1994b; Jakus et al., 2008). Similarly, the ability to become pregnant after experiencing an ectopic pregnancy is inversely related to the presence of antibodies to a conserved epitope expressed in both the chlamydial and human hsp60s (Sziller et al., 2008). The chlamydial hsp60 has recently been shown to also negatively affect pregnancy outcome by inducing apoptosis of trophoblasts (Equils et al., 2006).

The presence of Hsp60 in ejaculated semen and its correlation with anti-chlamydial IgA and antibodies to spermatozoa has also been demonstrated (Munoz et al., 1996). Similarly, anti-human hsp60 IgG has been identified in semen from male partners of infertile couples (Eggert-Kruse et al., 2002). Importantly, antisperm antibodies were shown to react with hsp70 present in a sperm membrane preparation (Bohring and Krause, 2003). Thus, immune responses to microbial heat shock proteins that are present within the male genital tract may also result in interference with successful fertilization.

AMNIOTIC FLUID

The amniotic cavity, within which fetal growth occurs, develops during the third week of gestation and becomes filled with fluids from both the embryo and the maternal circulation. Proteins and enzymes derived from the fetal respiratory, digestive and urinary tracts as well as immunoglobulin G (IgG) from the mother are present in the mid-trimester amniotic fluid (Underwood et al., 2005). In addition to its other roles in gestation, amniotic fluid also has an immune modulatory function. It must aid in the defense of the fetus against microorganisms that invade the amniotic cavity while at the same time prevent activation of a too vigorous pro-inflammatory immune response that may initiate the sequence of events leading to premature labor and expulsion of the fetus. Several recent studies have implicated the inducible 70 kDa heat shock protein (hsp70) as participating in intraamniotic immune functions. Human fetal membranes were induced to express hsp70 following exposure to lipopolysaccharide, a component of the cell wall of Gram negative bacteria (Menon et al., 2001). All mid-trimester amniotic fluids examined to date have been positive for hsp70. For some reason yet to be explained the median levels are more than twice as high in amniotic fluids from singleton pregnancies than from twin gestations (Perni et al., 2005; Jean-Pierre et al., 2006). The concentrations of hsp70 in amniotic fluid are positively correlated with the intraamniotic level of the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) suggesting that hsp70 either induces TNF-α or that hsp70 is induced following activation of an intraamniotic pro-inflammatory immune response. In further investigations, the addition of exogenous peptidoglycan, a component of the cell wall of Gram

positive bacteria, to *ex vivo*-cultured amniotic fluid resulted in the appearance of hsp70 into the culture supernatant. Thus, hsp70 expression is most likely a response to pro-inflammatory-inducing events and may aid in both recognition of a potential pathogen and modulation of the resulting immune response.

A recent study suggests that hsp70 may enter amniotic fluid in the form of exosomes (Asea et al., 2008). Exosomes were isolated from 23 mid-trimester amniotic fluids by differential centrifugation and banding at a density of 1.17 g/ml in sucrose gradients. All were positive for acetylcholinesterase activity and contained tubulin as well as the inducible and constitutive hsp70s. They were all negative for calnexin. Although the number of samples tested was limited, the intraamniotic exosome concentration was positively correlated with the number of pregnancies. Urine from newborns has been found to be positive for exosomes (Keller et al., 2007), suggesting that the exosomes in amniotic fluid may, at least partially, arise from the fetal kidney.

IgG antibodies to hsp70 have also been identified in amniotic fluid (Gelber et al., 2007). This raises the interesting possibility of hsp70-mediated cross-talk between the mother and fetus. The production of anti-human hsp70 antibodies frequently occurs as a consequence of a microbial infection. This may result from either the generation of antibodies to conserved epitopes of the microbial hsp70 or the production of antibodies directly to the human hsp70 as a result of its being a component of a microbial antigen-hsp70 complex. The transfer of these anti-hsp70 antibodies from an infected mother across the placenta and into the amniotic cavity will result in the formation of hsp70 antigen-antibody complexes within the amniotic fluid. Since the aggregation of hsp70 antigens by binding its homologous antibody results in enhanced hsp70-directed biological activity (Yokota et al., 2006), this antibody transfer will result in the potentiation of fetal intraamniotic hsp70 antigen-directed anti-microbial functions in response to a potential threat to the mother.

Several international investigations have examined whether detection of hsp70 or anti-hsp70 IgG during gestation has predictive value in terms of pregnancy outcome. In a study from Japan, elevated maternal serum hsp70 concentrations were associated with an increased rate of preterm delivery (Fukushima et al. 2005). A study from the United Kingdom demonstrated an association between levels of maternal anti-hsp70 IgG and the subsequent delivery of babies with a birth defect. It was suggested that formation of these antibodies was a consequence of elevated hsp70 antigen production during early pregnancy as a result of exposure to non-physiological conditions (Child et al., 2006). A study from Hungary reported an association between maternal circulating hsp70 protein concentration and development of preeclampsia (Molvarec et al., 2006). Women in China with a subsequent fetal demise or spontaneous abortion were reported to have had elevated levels of hsp70 in their peripheral blood lymphocytes obtained during their first trimester of pregnancy as compared to other pregnant women (Tan et al., 2007). A study from Switzerland concluded that the detection of IgM antibodies to hsp70 in fetal blood samples, obtained by cordocentesis at 22–25 weeks gestation, was highly correlated with the presence of

a fetal cytomegalovirus infection (Gerber et al., 2002). In contrast, IgM antibodies to hsp60 were non-informative.

CONCLUSION

In conclusion, heat shock protein expression and immunity are intimately related to gametogenesis, fertilization and embryo development. Whether the detection of alterations in expression of individual heat shock proteins and/or specific heat shock protein immunity will serve as biomarkers of specific mechanisms of infertility or post-fertilization failure or anomalies awaits further investigation. Once the involved mechanisms are more fully delineated it may be possible to apply exogenous interventions to prevent or minimize any adverse consequences. Conversely, the potential to manipulate heat shock protein expression for fertility control is a plausible, but totally unexplored, possibility

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CHAPTER 10

HEAT SHOCK PROTEINS AND DIARRHEA CAUSING MICROORGANISMS: EMERGENCE OF ENTEROAGGREGATIVE *ESCHERICHIA COLI*

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Abstract: Although a variety of bacterial species cause intestinal and extra-intestinal diseases in humans, the pathogenic *Escherichia coli* strains account for the majority of infections like typhoid, dysentery and diarrhea. These represent a notable burden particularly for children living in less developed regions of the world and are responsible for an estimated 780–900 million cases of diarrhea worldwide. These infections are benign and self-limiting, although young children are more prone than adults to severe complications from the disease. These diseases are usually acquired by ingesting food or water contaminated with human or animal feces and result in infection caused by bacteria, viruses, parasites, medications and food sensitivities. Enteroaggregative *Escherichia coli* (EAEC) infection, which generally presents with watery diarrhea and occasionally with blood and mucus is emerging as a particularly lethal pathogen responsible for acute and persistent diarrhea in both developing and developed countries. As pathogenicity becomes better elucidated and antigenic proteins or portions of proteins of the pathogens are identified, immunization becomes a feasible means of preventing diarrheal disease. Recently a great deal interest has been focused on the on 60-kDa (GroEL) and 70-kDa (DnaK) families since they represent major targets of the host's immune response. This chapter briefly describes current the role of HSP in diarrhea causing microorganisms and the emergence of EAEC as a global threat

Keywords: Diarrhea; bacteria; heat shock proteins

Abbreviations: AA, aggregative adherence; AAF, aggregative adherence fimbriae; AR, acid resistance; ASP, acid stress proteins; ATR, acid tolerance response; DAEC, diffusely adherent *Escherichia coli*; *E. coli*, *Escherichia coli*; EAEC, enteroaggregative

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Escherichia coli; EHEC, enterohemorrhagic *Escherichia coli*; EIEC, enteroinvasive *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; GSH, glutathione; HA, haemaglutination; HIV, human immunodeficiency virus; Hsp, heat shock proteins; *hsp*, heat shock protein gene; HSP, heat shock protein family; ID, infectious dose; LPS, lipopolysaccharide; MRHA, mannose-resistant haemaglutination; OMP, outer membrane proteins; ORT, oral rehydration therapy

INTRODUCTION

Diarrhea is a major public health problem throughout the world and is characterized by change in frequency and consistency of the stool. The massive dehydration which results from an infection will result in death unless prompt medication and appropriate fluid replacement are provided. A small proportion of these acute diarrheal episodes last for several weeks known as persistent diarrhea (Lanata et al., 1991). Acute diarrhea has a high fatality rate, which cannot be prevented by oral rehydration therapy (ORT) alone (Bern et al., 1992). Diarrheagenic *Escherichia coli* (*E. coli*) is responsible for infantile morbidity and mortality in developing countries (Smith et al., 1997) and has been classified into six classes; enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAEC) on the basis of distinct virulence properties and clinical syndromes (Nataro and Kaper, 1998). Enteroaggregative *E. coli* (EAEC) are increasingly recognized as an emerging pathotype responsible for acute and persistent diarrhea in both developing and developed countries (Bhan et al., 1986; Smith et al., 1997; Wallace-Gadsden, 2007). The long-term effects of this pathogen in developing countries may be more threatening than the short-term self-limiting diarrhea. EAEC has also been shown to be the major cause of persistent diarrhea among human immunodeficiency virus/acquired immunodeficiency syndrome (HIV) patients (Mayer and Wanke, 1995) and in traveler's diarrhea (Adachi et al., 2002, 2001).

Heat shock proteins (Hsp) are found in all cellular organisms. The most widely studied HSP in bacteria belong to the 60-kDa (GroEL) and 70-kDa (DnaK) families. As pathogenicity becomes better-elucidated and antigenic proteins or portions of proteins of the pathogens are identified, immunization becomes a feasible means of preventing diarrheal disease. Blocking the primary stages of infection, namely bacterial attachment to host cell receptors and colonization of the mucosal surface, may be the most effective strategy to prevent bacterial infections. A preclinical study with fimH adhesin (derived from uropathogenic *E. coli*) confirmed that immunoglobulin G (IgG) antibody alone, which transduced after parental vaccination with the fimH are sufficient to impede colonization, block infection and prevent disease (Wizemann et al., 1999). While IgG antibodies elicited against adhesins are protective, induction of immune responses along the mucosa can be augmented by a variety of antigen delivery systems that specifically target mucosa associated lymphoid tissue and activate the mucosal immune system (O'Hagan, 1998). These delivery systems include

whole inactivated or live-attenuated bacterial and viral vectors, biodegradable microspheres, liposomes and antigens conjugated to or co-administered with the cholera toxin B subunit or attenuated forms of heat labile toxin from *E. coli*. Unfortunately, there are no ETEC candidate vaccine shown to be effective in infants and young children in endemic areas (Svennerholm and Tobias, 2008). A great deal interest is currently centered on GroEL and DnaK families since they represent major targets of the host's immune response in *Staphylococcus aureus* (Qoronfleh et al., 1998). Also, GroEL- and GroES-like heat shock proteins (HSP) of *Campylobacter jejuni* have been shown to elicit a serum immunoglobulin G (IgG) as well as a secretory IgA response in experimentally infected rabbits (Wu et al., 1994) and immunization with proteins containing DnaK-specific sequences was demonstrated to protect against infection of mice with *Borrelia burgdorferi* (Bey et al., 1995). This chapter will briefly address the role of heat shock proteins in diarrhea causing microorganisms and cover the emergence of EAEC as a global threat.

CAUSES OF DIARRHEA: ROLE OF ACID STRESS

Once ingested, diarrhea-causing microorganisms endure exposure to acidic conditions in the intestine where the human colonic microflora is carrying out fermentation reactions from the carbohydrates, being taken as food. Although the pH of the intestinal contents is less acidic than that of the stomach but the presence of weak acids increase acid stress to potentially lethal levels for enteric bacteria (Salmond et al., 1984). The acidic pH of the human stomach is a daunting environment for these microorganisms and this low external pH results in the alteration of enzymatic functions and damage to various macromolecules in bacteria (Gorden and Small, 1993). Thus, acid stress can be defined as combined biological effect of low pH and weak acids present in the environment and therefore, acid resistance (AR) is perceived to be an important property to survive the acidity contributing to the pathogenesis of many enteric bacteria *i.e., E. coli* 0157:H7 (Lin et al., 1996), *H. pylori* (Mobley, 1997) and induction of virulence factors such as ToxR in *V. cholerae* (Miller et al., 1987). Outbreaks involving acidic foods *i.e.,* apple cider, dry fermented sausage, mayonnaise and yoghurt have drawn attention to the acidic tolerance properties of *E. coli* O157:H7 (Arnold and Kaspar, 1995). Over the past years, the existence of acid survival mechanism in Enterobacteriaceae have become apparent and have been studied most extensively in *Salmonella, Shigella* and *E. coli* (Lin et al., 1995).

The gastric juice is the first line of bactericidal barrier (pH 1.5) but the lowest pH where EAEC has shown to survival in vitro was at pH 4.0 only and not below it, which is comparatively higher than the stomach pH during the natural route of infection (Kaur, 2007; Kaur and Chakraborti, 2009). The acid challenge at pH lower than 4.0 (lowest pH for in vitro survival) can be correlated with infectious dose (ID), defined as a percentage of highly acid tolerant population (Brandl, 2006). During infection in stomach, the bacteria is already in stationary phase (non-dividing) and it is well reported that the survival potential of stationary phase or acid-adapted cells

is greater than that of exponentially growing cells over the initial period of acid challenge (Jordan et al., 1999). Once induced, the acid resistance system will remain active until cells reenter log phase (Lin et al., 1996). Therefore, the growth in vivo at pH 2.4 might compensate the survival of EAEC strain at pH 4.0 in vitro. Thus, the bacteria might be adapted when it encounters low pH in stomach.

Classically, pH homeostatic mechanism involving a series of protein antiport systems have been viewed as critical feature for surviving acid stress *E. coli* and *S. typhimurium* (Booth, 1985). The variety of amino acid decarboxylases available to *S. typhimurium* (lysine, ornithine and arginine) suggests that these microorganisms can survive extreme acid pH situations depending on which amino acids are present in the surrounding environment. Whereas *S. typhimurium* possesses only the acid tolerance response (ATR) systems, *S. flexneri* utilizes supplementation-dependent AR mechanism and *E. coli* has both types of systems (Lin et al., 1995). Cells actively growing at pH 7.7 rapidly die when shifted to conditions below pH 4.0. However, adapting these organisms to pH 5.8, a mildly acidic pH, for one generation increases their tolerance to more extreme acid conditions (pH 3.0) by inducible ATR, which is a two-stage process involving overlapping acid protection systems triggered at different levels of acidity. An inducible ATR has been demonstrated for the foodborne pathogen *Listeria monocytogenes* and the global effect of acid stress on protein synthesis has been reported (Phan-Thanh and Gormon, 1997).

The foundation of inducible acid tolerance is the induction of a set of acid shock proteins (ASP), there are 51 ASP for log phase ATR and 15 ASP for stationary phase ATR. At least some of these ASP are important for protecting the cells against extreme acid pH (pH 3.0) in minimal or complex medium (Lee et al., 1995, 1994). The regulation of porins synthesis is tightly controlled by environment and changes in the ratio of outer membrane proteins (OMP) C and F levels occur in response to pH, osmolarity and temperature (Heyde and Portalier, 1987). The outer surface proteins such as flagella and fimbriae found to be elaborated under acidic conditions (Walker et al., 1999). Acid shocked cells develop significant cross protection against other stresses including heat, salt, H_2O_2 **,** crystal violet and polymixin (Lee et al., 1995). In *E. coli*, the proteins induced by low pH (5.0) corresponded to four well-known HSP *e.g.,* GroEL, DnaK, HtpG and HtpM and three other pH induced proteins were the same as induced by osmolarity or anaerobiosis (Hickey and Hirshfield, 1990). The effects of pH are complex because they interact with other environmental factors also such as oxygenation, growth phase and various metabolites. The expression of acid-induced OMP was found to be growth phase dependent in EAEC strain (pH 4.0) with surprisingly the expression of two de novo OMP of sizes 41 and 48 kDa. Since these OMP were exclusively observed at pH 4.0, so considered as ASP (Kaur and Chakraborti, 2009). Several stress responses interact with the pH response including oxidative stress, heat shock and high osmolarity. Therefore, universal stress protein (USP) and Hsp antisera were used to confirm the function of acid-induced OMP in EAEC strain. In a recent study, three acid-induced OMP (25, 30 and 40 kDa) in EAEC strain showed cross reactivity with Hsp60 antisera (Kaur, 2007). Following acid-shock the synthesis of HSP was increased. Heat shock protein

GroEL is not restricted to heat shock protein Hsp65 alone but is a major acid shock protein also in *Brucella* (Lin and Ficht, 1995).

BACTERIAL MITOCHONDRIAL HSP

The mitochondrial 70-kDa chaperone (mtHSP70) is an essential component of the translocase of the inner membrane mitochondrial import complex, which binds preproteins on the matrix side of the inner mitochondrial membrane as importing motor for matrix proteins (Prinz et al., 2002). A cytosolic Hsp70 in *Cryptosporidium parvum* had similarity to all Hsp70 sequences with greatest scores to proteobacterial and eukaryotic mitochondrial Hsp70 (Emanuelsson and von Heijne, 2001). The Hsp70 and Cpn60 mitochondrial-type chaperones in *C. parvum* probably serve as a part of the fundamental elements for the import and maturation of many proteins, including Fe-S clusters (LaGier et al., 2003). In eukaryotes, the outer nuclear membrane is continuous with the rough ER, contributing to both protein synthesis and secretion (Palade, 1975). Interestingly, the nuclear envelope of the apicomplexan *T. gondii* appears to be an intermediate compartment for secretory trafficking from the ER to the Golgi apparatus (Hager et al., 1999).

HSP AND *Campylobacter jejuni*

Campylobacter jejuni is a bacterial enteric pathogen of increasing medical interest as a leading cause of infectious diarrhea throughout the world (Skirrow and Blaser, 1992). Only a few definite protein antigens have been characterized so far. One of the reconvalescent-phase sera usually react with the 65-kDa flagellin, the 44-kDa major outer membrane protein, and 25- to 29-kDa surface proteins (Dunn et al., 1987). PEB1, a bacterial adhesin for adherence to eukaryotic cells is well known for its immunogenic feature (Pei and Blaser, 1993), and *Campylobacter* trigger factor has recently been revealed to be a humoral antigen in the human host (Griffiths et al., 1995). GroEL- and GroES-like heat shock proteins (HSP) of *C. jejuni* have been shown to elicit a serum immunoglobulin G (IgG) as well as a secretory IgA response in the experimentally infected rabbits (Wu et al., 1994). HSP are synthesized in virtually all cells under conditions of stress, e.g., as a result of temperature or nutrient change. The best-characterized HSP belong to the 60-kDa (GroEL) and 70-kDa (DnaK) families and are the most conserved proteins known. Bacterial HSP have aroused the interest of microbiologists for many years, since they represent major targets of the host's immune response (Kaufmann and Schoel, 1994). Although less extensively studied than GroEL, DnaK homologues of many bacterial pathogens have been found to be immunogenic in humans or animals (Danilition et al., 1990). Furthermore, the experimental infection of mice with *Borrelia burgdorferi*, immunization with proteins containing DnaK-specific sequences may protect against microbial infection (Bey et al., 1995). Due to the extensive homology between bacterial HSP and their mammalian counterparts, the

humoral and/or T-cell response against these proteins has been proposed to influence the pathogenesis of autoimmune diseases (Kaufmann and Schoel, 1994). Since heat shock proteins are the promising candidates for subunit vaccines and therefore, efforts are needed to rule out the possibility for the vaccine design.

HSP AND *Salmonella*

The innate immune system recognizes the lipopolysaccharide (LPS) of *Salmonella*, an essential component of the bacterial outer membrane and a major determinant of *Salmonella* virulence (Chiu et al., 2004). Hsp70, Hsp90, CXCR4 and growth differentiation factor 5 (GDF5) have been identified as LPS receptor molecules or LPS associated proteins (LAPs) that play an essential role in delivery of an activation signal into the host cell, thereby triggering multiple signaling pathways including the pro-inflammatory responses (Triantafilou and Triantafilou, 2002). In addition to its role as a receptor for LPS, Hsp70 is also a molecular chaperone and under normal conditions, Hsp70, with the help of its co-chaperones (DNAJ proteins), is involved in protein folding and re-folding of misfolded proteins as well as intracellular protein transport (Kiang and Tsokos, 1998). Under stress conditions, Hsp70 can bind to damaged proteins and facilitate their refolding or target them for degradation (Samali and Orrenius, 1998). Hsp70 is functionally connected with the Hsp40 and HspH1 found to be induced in *S. choleraesuis* infected pigs, and gene expression was upregulated during the initial 48 h of infection. It has been reported that the treatment of cultured monocyte/macrophage cells with a *hsp*70 anti-sense oligonucleotide dramatically increased cell death in response to *S. choleraesuis* infection (Nishimura et al., 1997).

HSP AND ENTEROPATHOGENIC *E. coli* (EPEC)

Exposure of animals to thermal extremes and bacterial infections are stressors that increase metabolic activity with a resultant increase in ROS production (Palmer and Paulson, 1997). HSP are expressed in both prokaryotic and eukaryotic cells in response to a variety of stressors (Lindquist and Craig, 1988). Furthermore, the ubiquitous Hsp70 apparently is involved in mechanisms protecting the body from the deleterious effects of ROS (Polla et al., 1998). Interestingly, cellular glutathione (GSH) was found to be elevated under thermal stress conditions (Mitchell et al., 1983). Exposure of poultry species to mild stressors over a period of time enhances Hsp70 expression, but eventually, the birds become acclimated and no further increase in cellular Hsp70 can be demonstrated (Wang and Edens, 1998). Reports showed a strong relationship between thiol oxidation and Hsp70 synthesis in stressed cells (Mahmoud and Edens, 2003). It has been shown that chicken provided organic selenium in yeast are more resistant to thermal stress than chicken fed inorganic sodium selenite, and this was demonstrated by a lower expression of Hsp70. Selenium is an essential trace element that is involved in the regulation and

control of the body's antioxidant glutathione and GPx system, which plays a major role in the control of ROS (Palmer and Paulson, 1997).

The expression of Hsp70 is a classical sign of stress in animals because it is the physical manifestation of specific genes that are induced to combat stressors (Mahmoud and Edens, 2003; Polla et al., 1998). Studies have shown that broilers given diets without supplemental selenium had higher constitutive levels of Hsp70 than those given organic selenium as Sel-Plex. Additionally, EPEC challenge or acute heat distress in the animals have shown the inducible Hsp70 in greater concentrations. Both EPEC challenge and heat distress have the potential to increase ROS formation in association with elevation of Hsp70 in birds without supplemental selenium. However, with Sel-Plex, a lower level of Hsp70 expression indicated improved tolerance to the stress of EPEC infection. The hepatic content of GSH decreased dramatically after ethylene oxide treatment and that both *hsp*32 mRNA increased (approximately 40-fold) and *hsp*90 mRNA increased (approximately threefold) after high dose exposure to ethylene oxide (Katoh et al., 1991). The heat stress depletion of hepatic GSH was the greatest among broiler chicks without supplemental selenium followed by those supplemented with sodium selenite, but broilers given organic selenium showed the least depletion during heat stress (Mahmoud and Edens, 2003).

ENTEROAGRREGATIVE *E. coli* (EAEC)

The pathogenesis of EAEC was originally defined in persistent diarrhea in children in developing countries. Studies have shown that EAEC is the most prevalent pathotype among diarrhea caused by *E. coli* strains in Mongolian and Thai children (Sarantuya et al., 2004), in Austria (Presterl et al., 1999) and (Pabst et al., 2003) in Switzerland (Knutton et al., 2001). Outbreaks in Europe and Japan have also shown that EAEC is responsible for diarrhea (Itoh et al., 1997). EAEC has a potential cause of traveler's diarrhea in patients returning to UK from a variety of locations (Scotland et al., 1994), in Spanish travelers coming to developing countries (Vila et al., 2000), in European travelers to Guadalajara (Mexico), Ochio Rios (Jamaica) and Goa (India) (Adachi et al., 2001). A number of reports showed association of EAEC with immunocompromised patients (Durrer et al., 2000), Perhaps even more significant than the association of EAEC with persistent diarrhea are the data from Brazil and India (Kahali et al., 2004a). In the largest reported EAEC outbreak so far, 2697 (40.6%) Japanese children who consumed infected school lunches had severe diarrhea (Itoh et al., 1997). Strains of *E. coli* O44:H18 implicated in outbreaks in UK presumed to be EPEC, were actually in fact, EAEC (Smith et al., 1994). During a diarrhea epidemic in southern India (Pai et al., 1997), EAEC were identified in the stools of 11 of 20 persons who were thought to have outbreak related diarrhea.

The EAEC defining criterion is the aggregative adherence fimbriae (AAF) I, II and III regulated by a transcriptional activator called *aggR* for aggregative adherence to epithelial cells, HA and biofilm formation (Sheikh et al., 2001). However, each of the aggregative adherence fimbriae is present in only a minority of strains

(Kahali et al., 2004b). A three-stage model has been proposed for its pathogenesis: (1) abundant adherence to the intestinal mucosa, (2) increased production and deposition of mucus biofilm and (3) induction of mucosal inflammation (Huang and Dupont, 2004). The defining feature of EAEC is its ability to elicit characteristic "stacked brick" like aggregative adherence (AA) to HEp-2 cells for hemagglutination (HA) of human erythrocytes, clump and biofilm formation (Bhardwaj et al., 2006). Some of the EAEC isolates express chromosome-encoded virulence markers such as the 116 kDa secreted mucinase, Pic (a protein involved in intestinal colonization) (Henderson et al., 1999a). The second stage of pathogenesis of EAEC involves production of mucus containing biofilm (Tzipori et al., 1992), which leads to mucoid stools, malnutrition and persistent colonization. The final stage of EAEC pathogenesis involves an inflammatory response with cytokine release, mucosal toxicity and intestinal fluid secretion by enterotoxins and cause destruction of enterocytes (Henderson et al., 1999b). Plasmid encoded toxin (Pet), a serine protease as well as an autotransporter protein showed cytotoxic activity against cultured intestinal epithelial cells and erythrocytes (Villaseca et al., 2000). Some EAEC have also been found to produce α-hemolysin (Gomez et al., 1995). A number of OMP/afimbrial adhesins and fimbrial adhesins also play role in AA other than the AAF. The OMP size ranging from 30 to 43 kDa in different EAEC isolates like 38 kDa non-fimbrial protein for adherence (Wai et al., 1996) and a 16 kDa cryohemagglutinin, a major adhesin responsible for the 3 phenotypes namely mannose-resistant hemagglutinating activity (MRHA), clump formation and adherence to tissue culture cells and human intestines (Yamamoto et al., 1997) and dispersin forms a loosely associated layer on the surface of EAEC (Sheikh et al., 2002). Heterogeneity among EAEC strains has impeded its recognition for many years and its exact pathogenic mechanism is still unknown. If EAEC were sought in all laboratories, infection by this pathogen could explain over 25% of cases for which no pathogen was recovered (Adachi et al., 2001). A study in travelers to Mexico showed EAEC colonization but no symptom of diarrhea, which suggested that natural immunity to symptomatic EAEC diarrhea, could develop (Adachi et al., 2002).

CONCLUSION

The overall complexity of the stress response in enteric bacteria is probably greatly underestimated, since intracellular growth produces global patterns substantially different from those predicted by stress experiments under defined conditions (Abshire and Neidhardt, 1993). It appears that there are indeed numerous acid survival mechanisms; some have long-term dramatic effects while others have more subtle yet significant consequences. Continued study of acid survival in these organisms will provide general insights regarding stress management and will have a direct impact in understanding their pathogenesis. The apparent relevance of growth conditions to the expression of virulence determinants led us to investigate the effect of acid stress on the membrane composition in the microbes. A greater understanding of the

mechanism(s) employed by these organisms to sense and respond to acidic pH will lead to the development of drugs which can safely eradicate diarrhea.

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PART II

HEAT SHOCK PROTEINS AND PSYCHOLOGICAL STRESS

CHAPTER 11

HEAT SHOCK PROTEINS AND POST-TRAUMATIC STRESS DISORDER

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Abstract: Heat shock proteins (HSPs), a highly conserved family of stress response proteins, play a very important role in traumatic stress associated with Post-traumatic Stress disorder (PTSD), a psychiatric disorder observed in a high number of combat veterans and in others exposed to natural disasters, traffic accidents, terrorist attacks, etc. In this chapter, we will briefly review the expression of HSPs in the central nervous system (CNS) and discuss the underlying molecular mechanisms of HSPs in PTSD. We will also present evidence regarding the possible role of HSPs in glucocorticoid receptor (GR) trafficking and in the regulation of p11, a PTSD-associated protein. Advances in the understanding of the functions of HSPs have reached a point where clinical trials are warranted to determine the role of HSPs in the treatment and diagnosis of PTSD

Keywords: HSP; CNS; GR translocation; p11; mitochondrial function

Abbreviations: CNS, central nervous system; GR, glucocorticoid receptors; GRE, glucocorticoid response elements; PTSD, Post-traumatic Stress Disorder

INTRODUCTION

Heat shock proteins (HSPs), a highly conserved family of stress response proteins, play a very important role in traumatic stress associated with Post-traumatic Stress Disorder (PTSD), a psychiatric disorder. The HSPs are named according to their molecular weights. Hsp60, Hsp70, and Hsp90 (the most widely-studied HSP) refer to the families of heat shock proteins in the order of 60, 70, and 90 kDa in size, respectively. Traumatic stress-induced changes of HSP expression have been observed in the brain regions, where pathological changes in PTSD have been seen.

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For example, repeated (4-day) treatments with immobilization stress resulted in significant over-expression of Hsp60 and Hsp70 in the rat hippocampus (Hayase et al., 2003). In this brain region, HSPs co-localize with glucocorticoid receptors, indicating that HSPs play a role in the translocation of glucocorticoid receptors in neuronal cells. It is known that HSPs interact with the glucocorticoid receptors (GRs) and form HSP/GR complexes. These HSP/GR complexes translocate GRs from the cytosol to the neuronal nuclei where the GRs dissociate from the HSP/GR complex, bind to glucocorticoid response elements (GREs) in the target genes, including stress related genes, and initiate transcriptional activation. In addition, HSPs also translocate activated-GRs to the mitochondria, where GRs directly bind to mitochondrial membranes to regulate membrane potential and apoptotic protein release from the mitochondria. These changes of mitochondrial membrane potential and apoptotic protein release may be associated with neuronal cell death seen in PTSD hippocampus.

In this chapter, we will briefly review the expression of HSPs in the central nervous system (CNS), since their localization may be associated with their functions. Then, we will discuss the underlying molecular mechanisms of HSPs in PTSD. Specifically, we will provide evidence demonstrating the possible role of HSPs in GR trafficking and p11 regulation, a PTSD associated protein. Advances in the research of HSPs have shed light on the feasibility of the clinical application of HSP antagonists in PTSD, and warrant a clinical trail to determine whether HSP/GR is a therapeutic target for PTSD.

EXPRESSION OF HSPs IN CNS

Hsp90 Expression in the CNS

Expression of HSPs including Hsp90, Hsp70, Hsp60, and Hsp40 have been studied (D′Souza and Brown, 1998; Stacchiotti et al., 1997). In mammalian cells there are at least two Hsp90 isoforms, Hsp90α and Hsp90β which are encoded by separate genes. These ubiquitous and highly conserved proteins account for 1–2% of all cellular proteins in most cells. Hsp90 is part of the cells' powerful network of chaperones to fight the deleterious consequences of protein unfolding caused by non-physiological conditions. In the absence of stress, Hsp90 is a necessary component of fundamental cellular processes such as hormone signaling and cell cycle control. Hsp90 is differentially expressed in the various structures of the brain. In rats, Hsp90 mRNA is widespread but not ubiquitous, and labeling intensity varies among different regions. Hsp90 mRNA is abundant in limbic system-related structures, including the hippocampus, amygdala, mamillary body, piriform cortex, enthorhinal cortex, bed nucleus of the stria terminalis, medial habenular nucleus, and preoptic hypothalamic nuclei. The highest abundance of mRNA is seen in the Purkinje cell layer of the cerebellum, pineal body, choroid plexus, cerebellar granule cell layer, and cranial nerve nuclei. Moderate abundance of Hsp90 mRNA is observed throughout the

cortical gray mantle, the caudate-putamen, most other thalamic and hypothalamic nuclei, and the pontine. Weak Hsp90 mRNA signals were observed in the white matter fiber tracts and glial cells (Izumoto and Herbert, 1993). At protein levels, equal levels of Hsp90 is seen in the hippocampus, cortex, striatum, and cerebellum in neurons of the rat brain (Gass et al., 1994; Stacchiotti et al., 1997). At the ultrastructural levels, Hsp90 is predominantly expressed in the perikarya but to a lesser extent also in the dendrites and nuclei (Gass et al., 1994).

Hsp70 Expression in the CNS

Expression of the70 kDa Hsp (Hsp70) in the brain is also notable because the protein is highly inducible in glial cells and neurons following a wide range of noxious stimuli including ischemia, epileptic seizure, and hyperthermia. In animal studies, it is found that Hsp70 expresses differentially in the neuron and glia during the developmental stages. In postnatal day 4–5 cultures, Hsp70 mRNA expression was observed in astroglial and neuro-astroglial cultures, but it does not express itself in neurons (Voisin et al., 1996). At cellular levels, Hsp70 is associated with steroid receptors, actin, p53, polyoma T antigen, nucleotides, and other unknown proteins (Hermann et al., 2001; Kiang et al., 2003). Inducible expression of Hsp70 is present in many areas of the brain and is associated with cellular resistance to a variety of insults. For example, Hsp70 increased significantly in the brains of patients with Alzheimer's disease, and was localized exclusively in neuritic plaques and neurofibrillary tangles (Kakimura et al., 2002). Also, Hsp70 is involved in protective roles against thermal stress, cytotoxic drugs, and other damaging conditions.

Hsp70 mRNAs and proteins are diffusely localized over the nuclei of astrocytes and most microglia. Some cytoplasmic Hsp70 was observed in astrocytes of the mixed neuro-astroglial cultures. Hsp70 can be released by the glia and may enhance neuronal stress tolerance (Guzhova et al., 2001). In pharmacological studies, glucocorticoid-induced over-expression of Hsp70 in the cortex has been discovered. Pretreatment with prazosin, an alpha-1 receptor antagonist (Lacoste et al., 2001), resulted in significant attenuation of dexamethasone-induced expression of Hsp70 in the cerebral cortex. These data suggest that Hsp70 is not only expressed in the neuronal cells of the brain, but also has functions related to stress.

Hsp60 Expression in the CNS

The expression of another HSP, Hsp60, has also been examined in the central nervous system (D′Souza and Brown, 1998). Western blot analysis revealed constitutive expression of Hsp60 in postnatal development. Developmental profiles of Hsp60 suggest that it is also differentially regulated during postnatal development of the rat. Levels of Hsp60 show a developmental increase. Hsp60 is expressed in both the developing and fully differentiated neuron. At the cellular level, Hsp60 is located

in the mitochondria and assists in the folding of mitochondrial peptides. Like other HSPs, inducible expression of Hsp60 is seen in the brain and is associated with a variety of insults. A positive association between plasma Hsp60 and TNF alpha and a negative association with von Willebrand factor were found. There was also a significant association between elevated Hsp60 levels, low socioeconomic status, and social isolation, together with an association with psychological distress in women (Lewthwaite et al., 2002). Hsp60 is expressed in the gerbil hippocampal CA1 region and was induced by transient ischemia indicating its protective effect against ischemic damage (Hwang et al., 2007).

Hsp40 Expression in the CNS

Hsp40 is another HSP expressed in neuronal cells. Hsp40 highly concentrates in postsynaptic density (PSD) fractions. The staining of Hsp40 immunoreactivity distributed widely in the brain. Hsp40 immunoreactivity is seen in dendritic spines, especially in the subsynaptic web, with weak staining of PSDs. Hsp40 was also observed on the neuronal processes of cultured cerebral neurons. Expression of Hsp40 mRNA is low in the nonischemic mouse hippocampus, but is significantly up-regulated 4 h after ischemia (Tanaka et al., 2002). These results suggest that Hsp40 is localized at postsynaptic sites and postsynaptic chaperone activity may be mediated by this heat shock protein.

ROLE OF HSP IN GR TRANSLOCATION AND P11 GENE EXPRESSION

HSPs are expressed at low levels under normal physiological conditions in the CNS. Their up-regulation is sometimes described more generally as part of the stress response. Production of high levels of HSPs can be triggered by exposure to different kinds of environmental stress conditions, such as infection, inflammation, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light, among many others), starvation, hypoxia (oxygen deprivation), nitrogen deficiency (in plants), or water deprivation. HSPs function primarily as molecular chaperones, facilitating the folding of other cellular proteins, preventing protein aggregation, or targeting improperly folded proteins to specific degradative pathways. The current understanding of HSP functions is based on three main lines of evidence: (1) the role of HSPs in the clearance of waste proteins (Diller, 2006), (2) the role of HSPs in promoting the degradation of denatured proteins (van Noort, 2008), and (3) the role of HSPs in an array of cellular processes, including GR translocation (Filipovic et al., 2008). Here, we provide an updated view of the underlying molecular mechanism of HSPs in PTSD, the role of HSPs in GR trafficking and in the regulation of expression of p11, a PTSD associated gene.

Regulation and intracellular trafficking of the GR are critical determinants of GR action in both health and disease. The levels of Hsp70 protein changes in the hippocampus and the brain cortex of adult Wistar rat males exposed to acute

(immobilization, cold) and chronic (social isolation, crowding, daily swimming) stress or a combination of the two (Filipovic et al., 2005). Thermal stress induces expression of Hsp70 (Chen and Brown, 2007). In stressed-rats, cytosolic HSPs increase not only in the hippocampus, but also in the amygdala. Accompanying the HSP up-regulation, levels of HSP/GR and nuclei GR increased, indicating a role of HSPs in GR translocation under stressed conditions.

The HSP-regulated GR trafficking is dependent on the levels of glucocorticoid and GR activation, which are orchestrated by hypothalamic-pituitary-adrenal axis (HPA axis). HPA axis is proposed as one of the key circuit in pathogenic processes underlying PTSD. Over the last decade, many studies have shown abnormal HPA axis activity in PTSD, but these studies do not always report changes in the same direction (Yehuda et al., 1995; Lindauer et al., 2006). Both higher and lower concentrations of circulating glucocorticoids in PTSD patients have been reported. For example, Holocaust survivors with PTSD have low urinary cortisol excretion (Yehuda et al., 1995), while high early morning salivary cortisol levels have been reported in police officers with PTSD (Lindauer et al., 2006), and bereaved children suffering the death of a parent following the September 11, 2001 terrorist attacks had higher morning and 4:00 pm baseline cortisol concentrations than non-bereaved children (Pfeffer et al., 2007). The different stressors, the different methods used, the different patient populations recruited, and the different stages of the disorder examined in the various studies have been suggested as explanations for these diverse results.

In animal studies, traumatic stress induces a plasma glucocorticoid elevation (Vogel and Jensh, 1988), which regulates stress-related behavior (de Quervain et al., 1998; Adamec et al., 2006) and gene expression (Liberzon and Young, 1997; Roseboom et al., 2007). Acute restraint stress increases 5-HT7 receptor mRNA expression in the rat hippocampus (Yau et al., 2001). Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus (Meaney et al., 2000). Recently, a study demonstrated that dexamethasone (Dex), a synthetic glucocorticoid, can up-regulate p11, a S-100 calcium-binding protein (Gladwin et al., 2000; Huang et al., 2000), which was down-regulated in patients with depression (Svenningsson et al., 2006), a common co-morbid disorder in PTSD. These observations led to the hypothesis that traumatic stress may alter the expression of p11 in the brain and this alteration may be mediated by GRs, which are translocated by HSPs. Indeed, it is found that inescapable tail shock increased both prefrontal cortical p11 mRNA levels and plasma corticosterone levels in rats (Zhang et al., 2008; Ursano et al., 2008). Dex not only increased GR translocation, but also the up-regulation of p11 expression. p11 over-expression is mediated by GRs through the glucocorticoid response elements (GREs) within the p11 promoter. This p11 over-expression was attenuated by either RU486, a glucocorticoid receptor antagonist, or by mutating two of three glucocorticoid response elements (GRE2 and GRE3) in the p11 promoter (Table 1 and Figure 1). p11 mRNA levels were increased in postmortem prefrontal cortical tissue (area 46) of patients with PTSD (Figure 2). Inescapable tail shock also increased both prefrontal cortical Hsp90 and p11 levels in rats (Figure 3) (Zhang et al., 2008; Ursano et al., 2008).

1. Wild type and mutated sequences of the three GREs in the p11 promoter region

Oligonucleotide	Sequence[a]	Strand[b]	Nucleotide No.[c]
GRE[d]	GGTACA nnn TGTTCT		Webster (1999)
wt GRE[e]	tcTGTACA GGA TGTTCTag		Santa Cruz Biotechnology
GRE1	tcAGTAGA AAC GCACGTgc		–223 to 241
MutGRE1	tcAGTAGA AAC G**GTCC**Tgc	(+)	
GRE2	gcAAATGC AGA GGTAACcg		–354 to –372
MutGRE2	cgGTTACC AGA GGT**TCT**gc	(–)	
GRE3	gtGGCACG TGG ACACTTat		–427 to –445
MutGRE3	gtGGCACG TGG AG**TCC**Tat	(+)	

[a] 5′→3′, sense strand; n is any nucleotide. GRE sequence sites in the 15 bp palindrome are capitalized; flanking sequences are in lower case; and mutated (mut) bases are in bold type.
[b](+) refers to the sense strand and (–) to the complementary strand of oligonucleotide.
[c]Numbers refer to the nt position of putative GRE sites identified in the promoter by MatInspector (Genomatix Software GmbH, Germany). Flanking nucleotides are from the p11 promoter sequence (Huang et al., 2003).
[d]Consensus sequence for GRE elements (Chen et al., 2006).
[e]Consensus wild type (wt) GRE sequence, Santa Cruz Biotechnology.

The data obtained from our work in a rat model with inescapable tail shock, a p11-transfected cell line, and postmortem brain tissue from PTSD patients, outline a possible mechanism by which p11 is regulated by glucocorticoids elevated by traumatic stress. These results are consistent with others showing that GR needs to be translocated by HSPs from cytosol to the nuclei, where it activates the target gene promoter. Stress increases not only HSPs, but also p11. Indeed, regulation and intracellular trafficking of the GR are critical determinants of the role of GR in the regulation of p11 expression, which was over-expressed in the prefrontal cortex of both stressed rats and PTSD patients (Figs. 2 and 4). The data suggest that HSP/GR complex formation and GR translocation play important roles during the p11 expression process.

ROLE OF HSPs IN GR TRANSLOCATION AND MITOCHONDRIAL FUNCTION

PTSD is a common psychiatric disorder that displays morphologic changes in the hippocampus. Brain imaging studies have demonstrated a strong relationship between PTSD and a reduced volume of the hippocampus. Although the few available human MRI studies have not shown the reduced hippocampal volume to be temporally related to the onset of the stress/traumatic event, animal research has shown that the hippocampus can be damaged by stress hormones released during the ‘‘fear response’’, a primal reaction to imminent danger. Intensive stress is an apoptotic stimulus which increases cortisol levels and regulates expression of HSPs and GRs. Cortisol secretion increases not only in response to traumatic stress, but

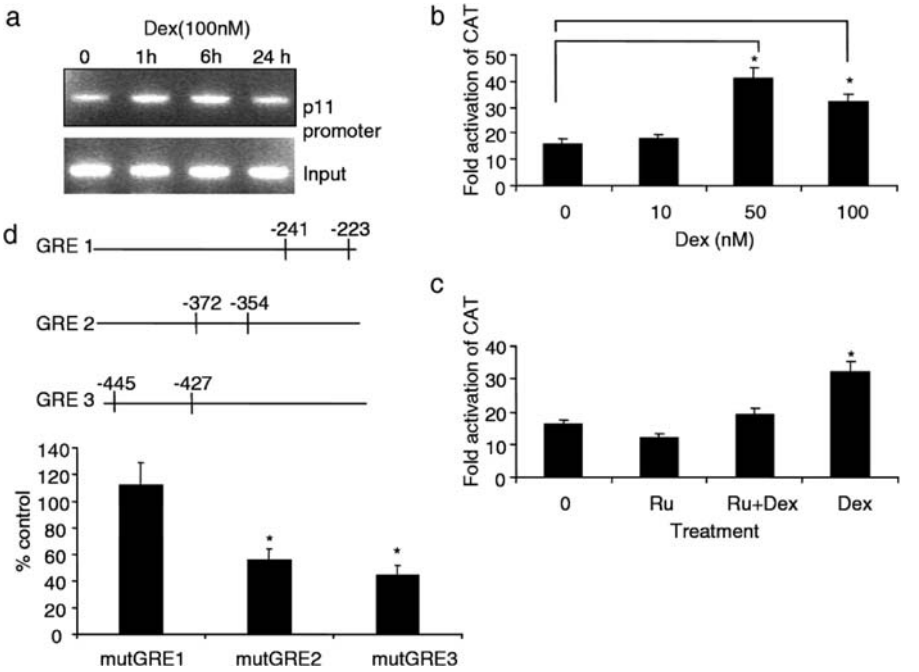

Figure 1. Effect of Dex on GR recruitment to the p11 promoter and p11 promoter activity. (**a**) GRE recruitment was determined by the ChIP assay in SH-SY5Y cells, which were incubated with 100 nM Dex for 0, 1, 6 and 24 h. (**b**) Dex increases p11 promoter activity in transiently transfected cells with pCAT-basic vectors containing the promoter from nucleotide +1499 to –89 in a dose-dependent manner. (**c**) RU486 blocks Dex-induced promoter activity and marginally decreases basal promoter activity. (**d**) Mutational analysis of GREs in the p11 promoter reveals that GRE2 and GRE3 are the two sites in the promoter that promote glucocorticoid-activated GR up-regulation of p11, since mutation at these sites, but not at GRE1, attenuates glucocorticoid-activated GR up-regulation. Bars are means±S.D. *Significantly different from similar condition without Dex. Data was analyzed by the Student's *t*-test

to any body stress, either physical (such as illness, trauma, surgery, or temperature extremes) or psychological. Elevated cortisol levels affect the functions of the mitochondria. For example, cortisone treatment results in a significant increase in mitochondrial volume in peripheral cells and decrease in the number of mitochondria per cell (Kimberg et al., 1968). High levels of circulating glucocorticoid hormones may be important mediators for elevating resting metabolic rates during severe injury or stress. The action of cortisol is much more complex. According to the common theory of steroid action, stress-induced increases in cortisol level may produce a genomic action, in which cortisol activated GR can be associated with several chaperone proteins-including HSPs such as Hsp90, Hsp70, Hsp60, and Hsp40 to form a GR complex. The complex then translocates from the cytosol to the nucleus of the cell. In the nucleus, GR directly binds to the glucocorticoid response element in DNA to regulate transcription, including the expression of BAX. The vital role of

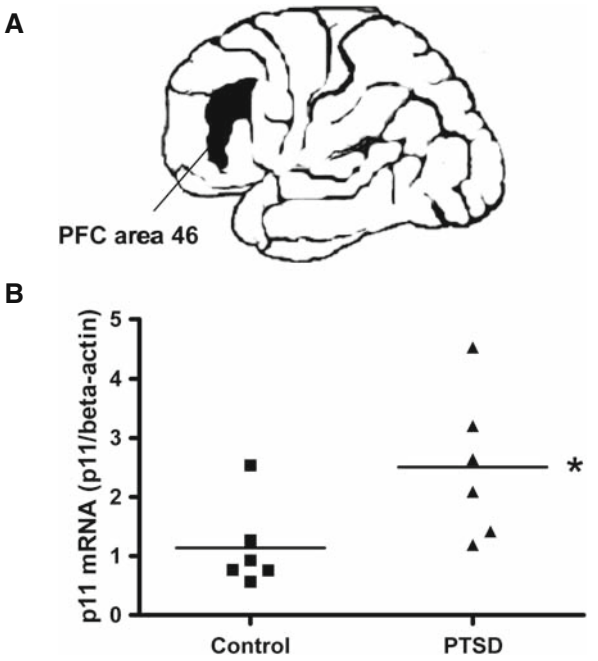

Figure 2. p11 mRNA expression in postmortem cortex is significantly increased in patients with PTSD compared with age- and sex-matched controls. (**a**) A perspective of the human brain to locate area 46 in the cortex. (**b**) All tissue blocks have the same (output) weight. cDNA was generated from 5 mg of total RNA for each sample to exclude the differences in RNA-content that could result from differences in sample weights. p11 mRNA is significantly greater in PTSD patients at postmortem compared to controls (n=6 per group, two cases died of suicide and four died of other causes in the PTSD group). Data is shown as means±S.E.M., $^*P$<0.05 (control vs. PTSD), and has been analyzed by the Student's t-test

BAX in apoptosis is well documented (Lindsten et al., 2005). Concurrent with the translocation of the BH3-only proteins to the mitochondria, BAX and Bak undergo conformational changes and oligomerization, presumably induced by transient interaction with the BH3-only protein (Korsmeyer et al., 2000). The oligomerized BAX and Bak may form a pore big enough for the apoptogenic proteins to pass through or they may destabilize the mitochondrial outer membrane through an unknown mechanism. Furthermore, BAX also binds to MAP-1 in mitochondria (Tan et al., 2005). This BAX-MAP-1 complex can produce a decrease of mitochondria membrane potential, which causes cytochrome c to be released from the mitochondria into the cytosol (Figure 5).

In contrast to genomic cortisol action, non-genomic cortisol effects (Falkenstein et al., 2000) are principally characterized by their insensitivity to inhibitors of transcription and protein synthesis, and represent the most obvious experimental evidence, their rapid onset of action (within seconds to minutes). Cortisol activates GRs, which directly bind to mitochondrial membranes. Rapid effects are likely to

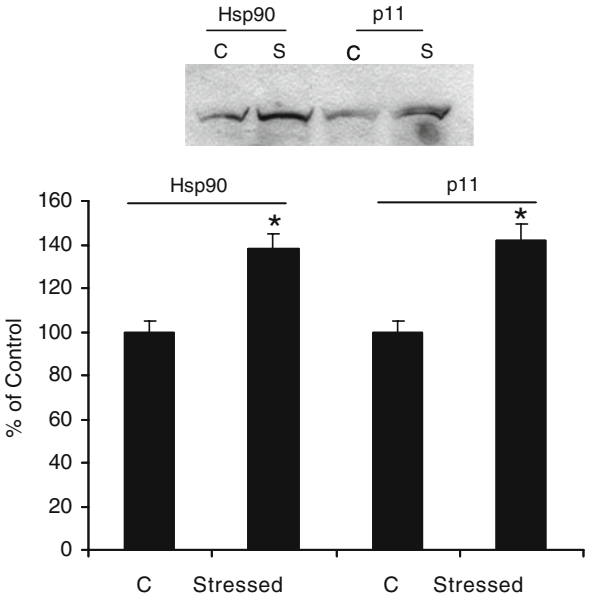

Figure 3. Effect of inescapable tail shock on protein expression of Hsp90 and p11 in rats. Inescapable tail shock significantly increased prefrontal cortical Hsp90 and p11 levels. $^{*}p<0.05$

be mediated through receptors as well. Both GR and BAX binding to mitochondrial membranes can produce a regulation of mitochondrial membrane potential. GR- or BAX-induced membrane potential changes can result in the release of cytochrome c from the intermembrane space of mitochondria to the cytosol, where it activates caspases and promotes apoptosis (Figure 5).

The role of mitochondrial cytochrome c in apoptosis has also been well documented. Cytosolic cytochrome c binds to Apaf-1, a cytosolic protein containing a caspase-recruitment domain, a nucleotide-binding domain, and multiple WD repeats (Zou et al., 1997). Apaf-1 binding of cytochrome c increases Apaf-1 affinity for dATP/ATP (Jiang and Wang, 2000). The binding of nucleotide to the Apaf-1/cytochrome c complex triggers its oligomerization to form the apoptosome, a multimeric Apaf-1 and cytochrome c complex (Zou et al., 1999).

In summary, stress-induced changes of mitochondrial membrane potential are mediated by the genomic and non-genomic actions of the cortisol. Such actions include the interaction between GRs and HSPs and the regulation of GR translocation and BAX expression (Figure 5). Therefore, HSPs play a very important role in the regulation of the function of mitochondria and cell death, which may be associated with the morphological changes of the hippocampus of PTSD patients. Understanding the molecular mechanism for stress-induced over-expression of HSPs, GR activation, and cell death in the hippocampus may shed new light on developing a mitochondrial membrane potential related therapeutic drug and/or diagnostic tool for PTSD.

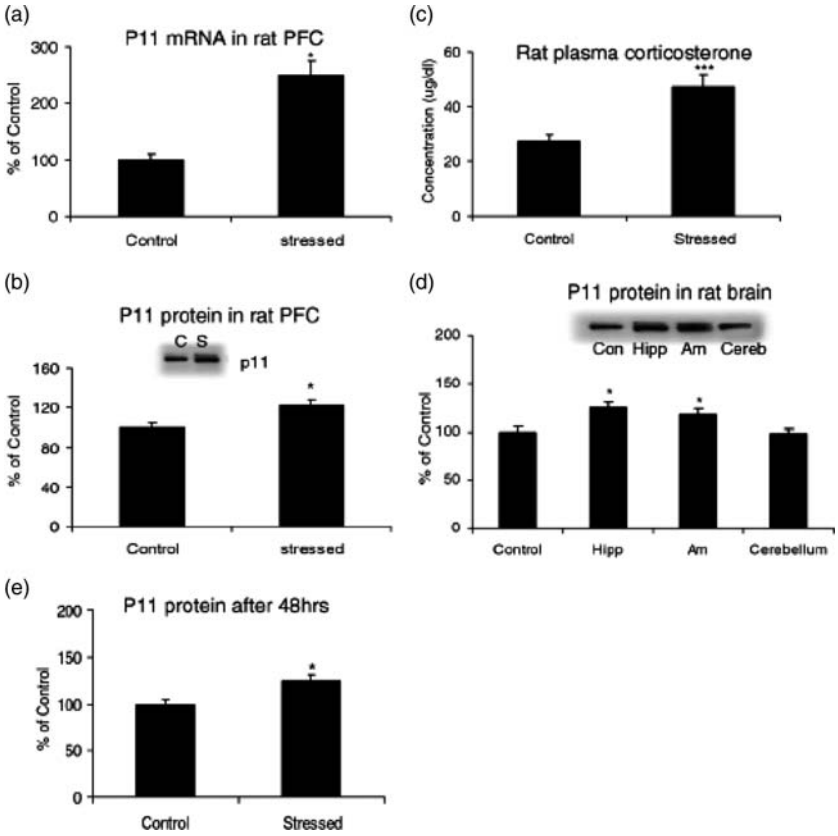

Figure 4. Stress induces p11 up-regulation in the PFC in rats at mRNA and protein levels and elevates plasma corticosterone levels. (**a**) Real-time PCR analysis shows that p11 mRNA levels increase about 2.5-fold on average in the stressed group. (**b**) Dot blot experiments reveal that p11 protein levels in the stressed PFC increase about 30% over control, consistent with the results of the real-time PCR results and indicating that stress increases p11 expression at both transcriptional and translational levels. (**c**) Stress increases plasma corticosterone levels in rats about 100%, as demonstrated by enzyme immunoassay. (**d**) p11 mRNA up-regulation also occurs in the hippocampus (Hipp) and amygdala (Am), but not in the cerebellum of stressed rats. (**e**) p11 protein level in the PFC of stressed rats is significantly higher at 48 h after shock treatment compared with control. The data was analyzed by Student's *t*-test; * $P<0.05$, *** $P<0.001$

CONCLUSION

HSPs have several main cellular tasks aimed at promoting neuronal function and inhibiting apoptotic activity. However, the detailed molecular mechanisms underlying their biological functions are still unclear. It is believed that HSPs, mitochondria, and other organelles may work coordinately to keep the cell in a stable and

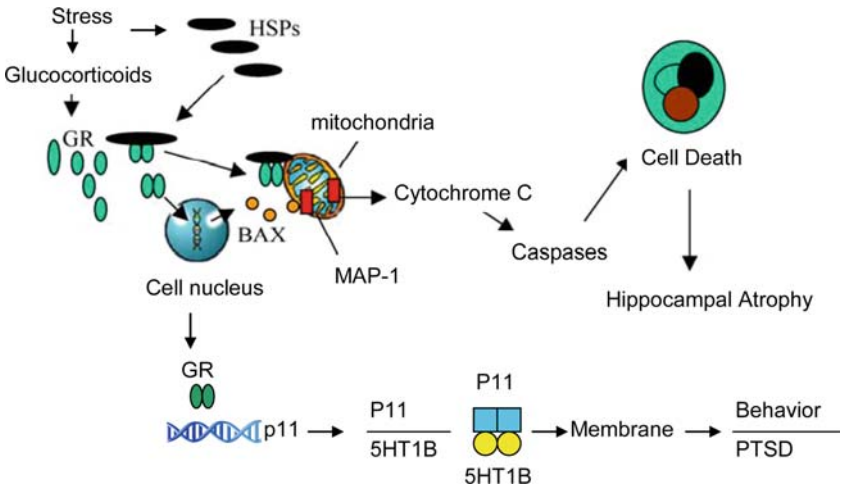

Figure 5. Role of HSP in stress-induced mitochondria membrane potential and hippocampual cell death. Stress stimuli result in the elevation of cortisone and activated GRs in the hippocampal neuronal cells. Apoptotic stimuli are transduced to the mitochondria through both genomic and non-genomic cortisol action. In the process of nongenomic action of the cortisol, activated GRs directly bind to the mitochondrial membrane, whose potential is regulated. This is followed by the genomic action of cortisol, in which HSPs are associated with GRs to form a HSP/GR complex. This complex is able to transport GRs into the nucleus, where GRs binds to GRE in DNA to produce transcriptional action. BAX is up-regulated by stress and transported to the mitochondria where it binds to MAP-1, resulting in a change of mitochondria membrane potential. The changes in mitochondria membrane potential induced by both non-genomic and genomic actions of the cortisol may cause cytochrome c to be released from the mitochondria into the cytoplasm, where cytochrome c promotes the action of caspases and apoptotic process. GRs also bind to the p11 gene at the GR response element of the p11 promoter region to up-regulate p11 expression, resulting in the formation of p11/5-HT 1B complex and transferring 5-HT 1B from cytosol to the membrane. Thus, GRs through p11 gene expression, may regulate translocation and function 5-HT 1B, which is associated with behavioral function and certain mental diseases, such as depression and PTSD

well-operated state. HSPs are particularly important in traumatic stress or PTSD and other neurodegenerative disorders because aberrant protein aggregation and neuronal cell death are the common pathophysiology of these disorders. Preliminary results have demonstrated an increase in the expression of HSPs, especially Hsp90, Hsp70, and Hsp60, and an increase of GR translocation in stressed rats. Such stress-induced changes resulted in neuronal cell death and p11 over-expression, and can be blocked by either GR antagonists or HSP antagonists. Thus, such intervention provides a promising therapy for PTSD patients. Advances in the research of HSP targets will shed new light on the feasibility of a clinical application of HSPs in PTSD. Future studies will focus on finding the mechanisms of HSPs in the regulation of GR translocation and p11 expression, searching for the selective HSP antagonists for the treatment of PTSD.

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CHAPTER 12

IN VIVO TISSUE SOURCE AND RELEASING SIGNAL FOR ENDOGENOUS EXTRACELLULAR HSP72

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Abstract: Environmental or emotional challenge triggers a cascading series of responses collectively termed the "stress response". Acute activation of the stress response modulates many aspects of physiology, including the immune system. Hans Seyle, the founding father of the concept of "biologic stress", proposed in the 1900s that tissues release "alarm signals" in response to local and/or systemic challenge, and that these signals can initiate and direct inflammatory responses. There is a renewed interest in the investigation of tissue alarm signals and such signals have been coined "danger signals", "DAMPS" (danger associated molecular patterns) and "hidden-self recognition signals". This chapter focuses on heat shock protein 72 (Hsp72) as one potential alarm signal. Activation of the systemic stress response and the release of norepinephrine from sympathetic nerve terminals, stimulates the release of endogenous Hsp72 into the blood. We hypothesize that the tissue source of in vivo released Hsp72 is the small intestine, and that NE binds to alpha1 adrenergic receptors expressed on intestinal epithelial cells triggering the release of Hsp72 into the blood. Furthermore, we hypothesize that lipopolysaccharide derived from endogenous gut microflora binds Hsp72, and that the extracellular Hsp72-LPS complex may contribute to stress-associated modulation of in vivo inflammatory processes

Keywords: Danger signals; inflammation; stress; alarm signals; bacterial translocation; sympathetic nervous system; β1-adrenergic receptors

Abbreviations: ADR, adrenergic receptors ; DAMPS, danger associated molecular patterns ; eHSP, extracellular heat shock proteins ; iHSP, intracellular HSP ; MHC, major histocompatibility complex ; NE, norepinephrine ; PBMC, peripheral blood mononuclear cells ; TLR, toll-like receptors

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INTRODUCTION

Environmental or emotional challenge triggers a cascading series of physiological responses that are collectively termed the "stress response". Acute activation of the stress response modulates many aspects of physiology, including the immune system. Hans Seyle who is the founding father of the concept of "biologic stress", proposed in the 1900s that tissues release "alarm signals" in response to local and/or systemic challenge, and that these signals can initiate and direct inflammatory responses (Seyle, 1936, 1978). There has been a renewed interest in the investigation of tissue alarm signals and such signals have recently been coined "danger signals" (Matzinger, 1994, 1998), "DAMPS" (danger associated molecular patterns), (Bianchi, 2007) and "hidden-self recognition signals" (Kono and Rock, 2008; Rock and Kono, 2008). The literature supports several endogenous molecules that can function as alarm signals when released into the extracellular environment, including high mobility group box 1 (HMGB1 (Aneja et al., 2008; Orlova et al., 2007), uric acid (Bianchi, 2007; Rock and Kono, 2008), galectins (Bianchi, 2007; Rock and Kono, 2008), cathelicidins (Bianchi, 2007; Rock and Kono, 2008) and heat shock proteins (Asea et al., 2000b; Bethke et al., 2002; Breloer et al., 2001; Campisi and Fleshner, 2003; Chen et al., 1999; Colaco, 1998; Fleshner et al., 2002; Habich et al., 2002; Moseley, 1998; Ohashi et al., 2000; Rock and Kono, 2008; Todryk et al., 2000; Vabulas et al., 2002)).

The focus of the current review is on one member of the 70-kDa Hsp (Hsp70) family of proteins, Hsp72. The Hsp70 family of proteins includes the constitutive 73-kDa protein (HSC73) protein and a highly stress-inducible 72-kDa protein (Hsp72) (Hartl, 1996; Morimoto, 1994). Intracellular Hsp72 is found in nearly every cell of the body and can be up-regulated after exposure to a variety of cellular and whole organism stressors (Hartl, 1996; Morimoto, 1994). Although basal concentrations of Hsp72 are low in most tissues, high concentrations of intracellular Hsp72 can be found in the absence of stressors in some tissues such as the frontal cortex (Heneka et al., 2003), pituitary (Campisi et al., 2003), adrenal (Campisi et al., 2003), brown fat (Matz et al., 1996, 1996b) and intestine (Arvans et al., 2005). Intracellular Hsp72 induction has been reported after exposure to a variety of whole organism physical stressors including heat (Kregel, 2002), tail shock stress (Campisi et al., 2003c; Nickerson et al., 2006), and restraint (Udelsman et al., 1993, 1991). Both males and females demonstrate robust Hsp72 responses after acute stressor exposure (Fleshner et al., 2006). Interestingly, induction of intracellular Hsp72 is not restricted to physical stressors. We reported that the experience of predatory fear, exposing rats to a cat with no physical contact, increases intracellular Hsp72 in the brain (Fleshner et al., 2004).

The goal of this chapter is to develop the following hypotheses: (1) activation of the systemic stress response triggers the release of endogenous Hsp72 into the blood; (2) stress-evoked norepinephrine (NE) released from the sympathetic nervous system is an important releasing signal of Hsp72; (3) a predominant tissue source of released eHsp72 is the gut; (4) NE released from sympathetic neurons innervating

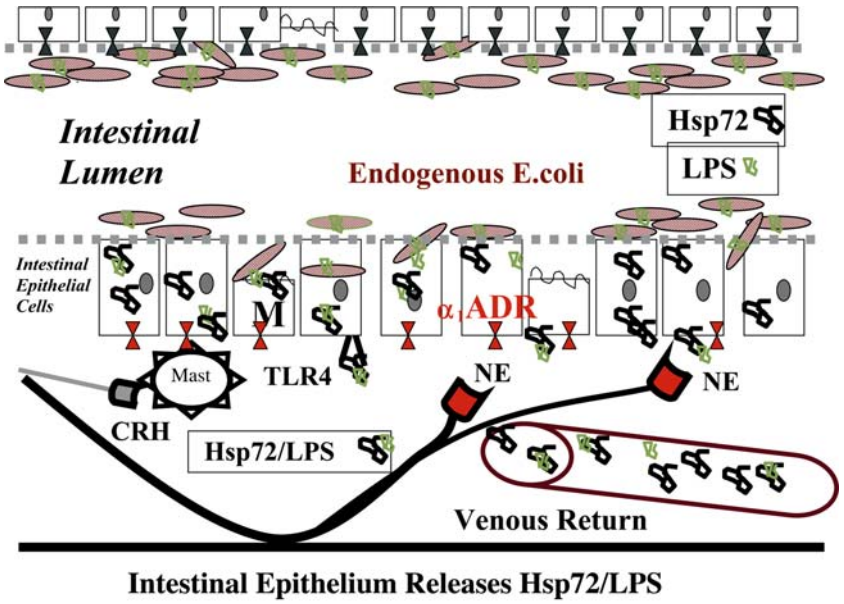

Figure 1. We hypothesize that activation of the acute stress response and sympathetic nervous system release of norepinephrine (NE) induces the release of eHsp72 and/or eHsp72/LPS into the blood via an α1-ADR mediated mechanism. Acute activation of the stress-response also stimulates the release of corticotropin releasing hormone (CRH) from nerve terminals in many tissues including the intestine. This can lead to bacterial translocation of endogenous enteric *E.coli* in part due to mast cell histamine release. Stress-induced release of eHsp72 and/or eHsp72/LPS complex could interact with CD14 and/or TLR4 receptors expressed on gut epithelia cells, further contributing to bacterial translocation. The eHsp72/LPS complex would enter the venous return of the small intestine and dump into the blood supply entering the mesenteric lymphnodes, spleen and liver.

the small intestine (duodenum, jejunum and ileum), binds to alpha1 adrenergic receptors (α1-ADR) expressed on gut epithelial cells and triggers Hsp72 release into the venous return; (5) lipopolysaccharide derived from endogenous gut microflora binds eHsp72; and (6) the eHsp72-LPS complex may contribute to stress-associated modulation of in vivo inflammatory processes. Schematic representation of these ideas if illustrated in Figure 1.

STRESS AND EXTRACELLULAR HSP72

The first reports that eHsp72 was detectable in the circulation of humans were published by Pockley and colleagues in 2000. This group reported that people suffering from a variety of disease states such as renal disease (Wright et al., 2000), hyptertension (Pockley et al., 2002), and atherosclerosis (Pockley et al., 2003) have chronically elevated basal levels of eHsp72 relative to healthy aged-matched

controls. In addition to elevated basal eHsp72 associated with disease pathology, Dybdahl et al. (2002) reported patients with coronary artery disease have an acute increase in eHsp72 in response to the stress of coronary bypass surgery. The role of eHsp72 in disease states has recently been reviewed (Ciocca and Calderwood, 2005). Not long after these reports, we (Campisi and Fleshner, 2003; Campisi et al., 2003b; Fleshner et al., 2003) and Febbraio et al. (Febbraio et al., 2002; Walsh et al., 2001) reported that organisms in the absence of clinical disease states also rapidly increase the concentration of eHsp72 in blood after exposure to acute physical and/or psychological stressors. These papers were the first to demonstrate that an increase of eHsp72 in the blood occurs in healthy organisms after exposure to acute stressors.

RELEASING SIGNALS AND SECRETORY PATHWAYS OF EXTRACELLULAR HSP72: OVERVIEW

It was first suggested that Hsp72 is released from cells as a result of only necrotic/lytic cell death (Gallucci et al., 1999). While it is true that necrotic cell death can cause the release of eHsp72 (Basu et al., 2000; Berwin et al., 2001; Sauter et al., 2000), it is now recognized that elevated eHsp72 may be found in the absence of necosis. In fact, glial cells (Guzhova et al., 2001), B cells (Clayton et al., 2005) and tumor cells (Abboud et al., 2008; Gastpar et al., 2005) have been shown to exocytotically release eHsp72 in a non-classical protein transport pathway (Abboud et al., 2008; Broquet et al., 2003; Gastpar et al., 2005; Hightower and Guidon, 1989; Lancaster and Febbraio, 2005) that may involve exosomal packaging of Hsp72 (Clayton et al., 2005; Guzhova et al., 2001; Lancaster and Febbraio, 2005). Furthermore, numerous whole organism stressors have been observed to elevate circulating eHsp72 within 10–25 min of stressor onset (Campisi et al., 2003b), a speed that suggests the classic protein induction/necrosis release pathway is not likely. In addition, eHsp72 is found in the blood after exposure to stressors that are unlikely to result in large necrotic cell death, such as conditioned contextual fear, predatory stress, and exercise stress (Fleshner et al., 2006). Thus, while necrotic cell death can result in the extracellular release of cytoplasmic Hsp72, accumulating evidence supports the existence of other factors such as exosomal release and hormonal receptor-mediated exocytotic pathways.

RELEASING SIGNALS AND SECRETORY PATHWAYS OF EXTRACELLULAR HSP72: EXOSOMAL RELEASE

It has recently been reported that Hsp72 may be released within exosomes. Exosomes are membrane vesicles that form within multivesicular bodies (MVB) and are secreted from cells when the membrane of the MVB fuses with the plasma membrane and the internal vesicles become extracellular. Since the primary role of membrane vesicles within MVB are to sort and distribute their constituents to the appropriate destination, exosomes contain various signaling molecules that

have been designated for release. These molecules are already known to commonly include various immunomodulating substances such as major histocompatibility complex (MHC) class I and II, CD80 (B7.1), CD86 (B7.2), and CD54 (ICAM-1), and now several researchers have demonstrated that Hsp72 are also contained within exosomes. It should be noted that membrane vesicles within MVB could have other fates such as fusion with a lysosome that results in the degradation of proteins, or fusion with the plasma membrane resulting in the expression of proteins on the cell surface. The surface expression of Hsp72 is thought to be important in cell-to-cell interactions and the recognition of tumors (Bausero et al., 2005; Gastpar et al., 2005). Although this is an exciting feature of Hsp72-immune interactions, cell surface Hsp72 function is beyond the scope of the current chapter.

The association of Hsp72 with exosomes has been reported in a variety of cell types including B-cells, tumors, and human peripheral blood mononuclear cells (PBMC), although there appears to be some cellular specificity of Hsp72 expression and release within exosomes. For example, Hsp72 was only detected within the lumen of exosomes in B-cells, but was expressed on the exosome surface and lumen in tumor cells. In addition, interferon-gamma induced excretion of Hsp72-containing exosomes in tumor cells could be prevented by disruption of lipid rafts, but the same disruption in PBMC had no effect on the heat-stress induced release of Hsp-containing exosomes. Thus, the necessity of lipid rafts may be either cell type or stimulus dependent. Furthermore, heat-stress increased the quantity of exosomes released from B-lymphoblastoid cells, while only increasing Hsp72 levels within exosomes and not number of exosomes in PBMC. This suggests that cells can use one of two mechanisms (or both) to increase eHsp72 levels via exosomal release; (1) increase the rate of exosomal release, (2) increase the concentration of Hsp72 within each released exosome.

RELEASING SIGNALS AND SECRETORY PATHWAYS OF EXTRACELLULAR HSP72: ADR-MEDIATED RELEASE

Currently, it is unknown whether stress-induced elevations in circulating eHsp72 are contained within or expressed on the surface of exosomes. Recent data from our laboratory, however, have determined that α1-ADR play a critical role in triggering the elevation of circulating eHsp72 during times of stress (Johnson et al., 2005). Johnson et al. (2005) reported that prazosin (an α1-ADR antagonist) blocked the stress-induced increase in circulating eHsp72 concentration in laboratory rats. This is particularly interesting because α1-ADR activation is known to induce a Ca^{2+} influx, an intracellular signal that has been observed to trigger the release of exosomes from many cell types including gut epithelial cells (Baglole et al., 2006, 2007). In addition, since α1-ADR stimulation is known to increase intracellular Hsp72 levels in many cell types, activation of α1-ADR may increase the amount of Hsp72 stored within each exosome, similar to that observed after heat-stress in PBMC. Since norepinephrine binds with higher affinity than epinephrine to α1-ADRs (Hardman

and Limbird, 2001), and adrenalectomy, which depletes ~95–99% of epinephrine (Hessman et al., 1976; Vollmer et al., 1995) has been shown to have no effect on stress-induced eHsp72 release after stress (Johnson et al., 2005), we hypothesize that the increase in circulating eHsp72 during stressor exposure is due to sympathetic nervous system activation and the release of norepinephrine that acts at α1-ADR to stimulate the release of Hsp72.

IMMUNE MODULATORY EFFECTS OF EHSP72

The eHsp72 clearly modulates immunity. The function of in vivo endogenous eHsp72 is likely dependent on its cellular source and, consequently, its associated peptides. There is evidence, for example, that Hsp72 is released or expressed on the cell surface of cancer cells (Botzler et al., 1998a; b; Calderwood et al., 2006; Multhoff et al., 1995), B cells (Clayton et al., 2001, 2005), monocytes (Abboud et al., 2008; Aneja et al., 2006), and glial cells (Guzhova et al., 2001). One can envision unique immunological effects of Hsp72 derived from neoplastic cells and associated peptides, versus Hsp72 derived from normal healthy cells and associated peptides. In addition, eHsp72 can bind a variety of receptors (Calderwood et al., 2007a) and produce an array of immunological consequences that range from modification of antigen specific acquired immune responses (i.e., dendritic cell antigen cross presentation (Delneste et al., 2002; Li et al., 2002), facilitated anti-cancer immunity (Srivastava, 2002; Srivastava and Heike, 1991), auto-immunity (Millar et al., 2003)) to regulation of innate pro- and anti-inflammatory responses (Quintana and Cohen, 2005; van Eden et al., 2005; Wang et al., 2005). Pittet et al. (2002) reported that humans who experienced trauma had increased serum levels of eHsp72, and that higher levels of eHsp72 correlated with improved survival. More recently this same group reported that higher concentrations of eHsp72 in pulmonary edema fluid was predictive of preserved alveolar epithelial clearance and potentially better survival following acute lung injury (Ganter et al., 2006). Thus in these studies, eHsp72 functioned to modulate the inflammatory response in an adaptive fashion.

Based on primarily in vitro studies, eHsp72 can robustly stimulate inflammatory cytokine production and other immune responses (Asea et al., 2000b; Breloer et al., 2001; Multhoff et al., 1999). We, and others, have reported that eHsp72 in vitro stimulates inducible NO synthase (Panjwani et al., 2002), NO (Campisi and Fleshner, 2003), TNF-α (Asea et al., 2000b; Campisi and Fleshner, 2003), IL1β (Asea et al., 2000b; Campisi and Fleshner, 2003), and IL-6 (Asea et al., 2000b; Campisi and Fleshner, 2003) production from macrophages and neutrophils. Hsp72 also stimulates DC cytokines and chemokine production as well as dendritic cell (DC) maturation (Lehner et al., 2000, 2004; Wang et al., 2002, 2005). Interestingly, eHsp72 can also stimulate anti-inflammatory cytokines (Pockley, 2003; van Eden et al., 2005; Wang et al., 2005) and has been reported to desensitize or reprogram LPS mediated pro-inflammatory responses in THP-1 cells (a monocytic leukemia cell line (Abboud et al., 2008; Delneste et al., 2002)).

In a series of elegant studies by Thomas Lehner and colleagues, unique immunological consequences of Hsp72 can be localized to specific domains of the Hsp72 molecule. For example, the C-terminal portion of Hsp72 (aa359-610) stimulates production of chemokines, IL-12, TNF-α, and NO; induces Th1 polarization and stimulates the maturation of DC. The N-terminal ATPase portion (aa 1-358) largely lacks these functions (Lehner et al., 2000; 2004; Wang et al., 2002, 2005). The C-terminal portion of several species of Hsp72 (microbial and human) binds to CD14, TLR4, scavenger receptors (Calderwood et al., 2007b) and CD40 on antigen presenting cells (Wang et al., 2001). CD40-CD40L interactions made between antigen presenting cells (APC) and T cells serves an important co-stimulatory role. Thus Hsp72 may function to both stimulate innate immunity via CD14 and TLRs and facilitate T cell responses via activation of CD40 expressing APCs. This makes Hsp72 a molecule positioned to play an important role at the interface between innate and adaptive immunity (Wang et al., 2005).

Evidence of a cell surface receptor for Hsp70 has been characterized on several cell types including macrophages/neutrophils (Asea et al., 2000a, 2002; Reed and Nicchitta, 2000; Sondermann et al., 2000), B cells (Arnold-Schild et al., 1999) NK cells (Gross et al., 2003; Multhoff et al., 2001) and epithelial cells (Theriault et al., 2005). A number of cell-surface binding proteins for eHsp have been implicated including CD91, TLRs, scavenger receptors, CD40 and CCR5 (reviewed in Calderwood et al., 2007a). Toll-like receptor-2 (TLR2), TLR4, and CD14 clearly play a critical role in transducing eHsp72 inflammatory signals to innate immune cells (macrophages/dendritic/neutrophils) supports the role of (Asea et al., 2000b, 2002; Vabulas et al., 2002; Visintin et al., 2001). Mammalian Toll-like receptors are transmembrane proteins that are evolutionarily conserved between very primitive organisms (such as insects) and humans (Akira et al., 2001). It has been suggested that just as released eHSP may function as "danger signals" or "messenger of stress" to the immune system, the TLRs may function as surveillance receptors for those signals (Johnson et al., 2003). In addition, exposure to prior injury stress was recently reported to produce a long-term (1–7 days) potentiatation of TLR2 and TLR4-induced IL1β, IL6 and TNF-α production by spleen cells (Paterson et al., 2003), and chronic social stress (Avitsur et al., 2003) modulates TLR4-mediated responses. These data support the hypothesis that stress-induced modulation of immune function may involve TLR2 and TLR4. Extracellular Hsp72 exerts it effects on innate immune cells by stimulating the inflammatory MyD88/IRAK/NF-kappaB signal transduction pathway (Vabulas et al., 2002). A rapid intracellular Ca^{2+} flux ensues within 10 s of eHsp72 binding with high affinity to monocytes or macrophages (Asea et al., 2000b). This is important because it distinguishes eHsp72 signaling from LPS signaling, that does not induce Ca^{2+} flux (McLeish et al., 1989). Based on work by Asea and colleagues (Asea et al., 2000a, b; Asea et al., 2002), eHsp72-induction of NF-kappaB and inflammatory cytokines requires the expression of CD14, in addition to TLR2 and TLR4. Thus the variety of immunological functions modulated by Hsp72 is likely dictated by (1) cell type releasing the Hsp72 plus consequent peptide, and (2) the cell type and specific receptor binding the eHsp72.

Interestingly, Asea and colleagues have proposed that CD14 could function as a co-receptor for eHsp72 (Asea et al., 2000b). It this is true, then eHsp72 released into the blood after exposure to psychological and/or physical stressors may result in optimal stimulation of the inflammatory cascade only in the presence of CD14 activation. Interestingly, binding CD14 plus either TLR2 and/or TLR4 with selective receptor agonists (Pam3Cys binds TLR2 or Taxol binds TLR4) resulted in synergistic increases in NF-kappaB (Asea et al., 2002). Thus, facilitation of innate immune responses by eHsp72 after exposure to stress may be restricted to cells that express CD14 and/or are binding bacteria, LPS, or eHsp72 bound to endogenous LPS released from the gut microflora. In many instances, therefore, we hypothesize that acute-stress induced release of eHsp72 may have little or no effect on innate immune cell production of nitric oxide (NO) and/or inflammatory cytokines. If, however, eHsp72 is complexed to endogenous LPS, the host is challenged with a pathogen (e.g., bacteria) or already suffers from chronic inflammatory disease (e.g., atherosclerosis, Alzheimer's disease, inflammatory bowl disease), eHsp72 could extravasate from the blood into the inflamed tissues and bind to macrophages and/or neutrophils. Cells that are binding bacteria (LPS) and eHsp72 via CD14 and/or TLR2 or TLR4 could have potentiated NO and/or cytokine response, resulting in facilitated bacterial killing (Campisi et al., 2003a; El Mezayen et al., 2007) and/or exacerbation of chronic inflammatory disease.

EHSP72 AND LPS: IN VITRO VERSUS IN VIVO

One issue that remains a source of debate is whether macrophage activation and/or inflammatory cytokine stimulation induced by Hsp72 reported in earlier in vitro studies is actually due to Hsp72 or is instead a result of LPS contamination inherent in the recombinant protein used. Many studies have carefully demonstrated that (1) stimulation of inflammatory cytokines and NO by eHsp72 in vitro is specific to eHsp72 and is not due to non-specific effects of endotoxin contamination in the recombinant Hsp72 protein, and (2) that the intracellular signaling pathways used by lipopolysaccharide (LPS) versus Hsp72 are unique and distinguishable, (Asea et al., 2000b; Campisi and Fleshner, 2003; Panjwani et al., 2002). Nonetheless this issue deserves discussion.

Gao and Tsuan (Gao and Tsan, 2003a, b; Tsan and Gao, 2004) have published several articles warning that researchers must consider the contribution of LPS contamination inherent in recombinant heat shock protein when testing the immune function of these proteins in vitro. One observation that is reported to support this idea is that endotoxin free recombinant human Hsp72 (rhHsp72) does not stimulate inflammatory cytokines from murine macrophages in vitro (Gao and Tsan, 2003a). We have replicated these observations (Johnson and Fleshner, 2006) and agree with the conclusion that in vitro testing of the immunological function of rhHsp72 must be conducted with caution. These results, however, do not diminish our hypothesis

that endogenous Hsp72 released into the blood during an acute stress response has in vivo immunomodulatory functions.

In fact, these findings have stimulated a new series of studies investigating which peptides are associated with eHsp72 released into the circulation and if those peptides work in concert with the Hsp72 to stimulate immune function. It seems clear that endogenous Hsp72 released into the blood in vivo likely is not "naked". In fact it has been previously suggested that considering the complex forming ability and chaperoning function of Hsp72, in vivo "endotoxin-free" Hsp is probably non-existent (Prohaszka and Fust, 2004). This observation and the fact that exposure to a variety of physical and psychological stressors stimulates commensal bacterial translocation from the gut (Deitch and Berg, 1987; Horton, 1994; Katafuchi et al., 2003; Nazli et al., 2004; Nettelbladt et al., 2003; Velin et al., 2004), have led us to hypothesize that some or all of the endogenous eHsp72 released by acute stress into the blood may be, in fact, associated with LPS released from endogenous gut bacterial flora. Perhaps it is this Hsp72/LPS complex that extravasates across the leaky vasculature or is transported from the blood into the inflamed tissues to either facilitate host antimicrobial defenses killing (Campisi et al., 2003a; El Mezayen et al., 2007) or exacerbate inflammatory disease processes.

Interestingly, Quintana and Cohen (2005) recently reviewed this issue and concluded that the in vitro work by Gao and Tsan does not negate the evidence that endogenous HSP manifest innate immune activities. In fact they suggest that endogenous eHSP may perform diverse functions via two modes of inflammation: sterile inflammation that is triggered by endogenous HSP and is necessary for body homeostasis, wound healing, angiogenesis, regeneration and immunoregulation, and septic inflammation that is triggered by the release of microbial HSP. Quintana and Cohen (2005) also suggest that eHsp72 may bind to LPS and augment immune responses by facilitating the transfer of LPS to the TLR4-MD2 leading to improved signal transduction and inflammatory cytokines responses. Our work supports several aspects of these ideas. Specifically, we have evidence that activation of the acute stress responses results in the release of endogenous eHsp72 and that eHsp72 is not "naked" and instead is found in the blood bound to LPS (Fleshner et al., 2007).

STRESS, BACTERIAL TRANSLOCATION AND THE EHSP72/LPS COMPLEX

We propose that the eHsp72 in the blood after exposure to an intense acute stressor may be bound to LPS derived from communal enteric bacterial translocation (Figure 1). This hypothesis is plausible in part because there is strong evidence that activation of the stress responses can induce translocation of enteric bacterial. Movement of enteric bacteria from the gastrointestinal lumen through the intestinal mucosal epithelial barrier is referred to as bacterial translocation. This process can occur in two ways, either via transcellular passage, movement of bacteria through

epithelial cells, or via paracellular passage, movement of bacteria between adjacent epithelial cells. During normal physiological conditions, adjacent epithelial cells are connected by tight junctions that prevent the passage of bacteria between cells thus only transcellular passage of bacteria occurs and this process is mediated by specialized mucosa epithelial cells called microfold or M cells. M cells are interspersed in the follicle-associated epithelia, part of the epithelia that is association with gut-associated lymphoid tissue (GALT) (Gebert et al., 1996). The apical surface of M cells have specialized receptors that enhance their interaction with luminal bacteria and mediate the transcytosis of bacteria to immune cells contained with the underlying GALT (Giannasca et al., 1999; Neal et al., 2006; Tyrer et al., 2006). The immune response within GALT is predominantly mediated by the release of transforming growth factor-beta and promotes immune tolerance by suppressing inflammatory responses (McGuirk and Mills, 2002).

Exposure to a wide number of stressors such as, restraint (Ando et al., 2000; Dunn et al., 2003), social disruption (Bailey et al., 2006), water avoidance stress (Velin et al., 2004), and sleep deprivation (Bergmann et al., 1996), increase bacterial translocation across the mucosal epithelial barrier. One consequence of translocation is that live, replicating bacteria can often be cultured from tissue homogenates of mesenteric lymph nodes, spleen, and liver. In fact, bacterial translocation is thought to be a critical factor involved in morbidity and mortality following some stressors such as burn injury (Gianotti et al., 1996; Sittig and Deitch, 1988), heat stroke (Gathiram et al., 1987, 1988a), and ischemia-reperfusion injury (Gathiram et al., 1988b). As described above, bacteria translocation can occur either via transcellular or paracellular passage and during times of stress it appears bacteria translocation occurs via both routes. Some data suggest transcellular permeability may be more sensitive to disruption by stressor exposure compared to paracellular permeability. This is based on the findings that acute mild stressor exposure increases the passage of HRP molecules through epithelial cells but the epithelial layer remain impermeable to ^{51}Cr-EDTA (a paracellular marker molecule), while chronic mild stressor exposure increases the passage of both HRP and ^{51}Cr-EDTA molecules (Ferrier et al., 2003; Velin et al., 2004). The mechanisms by which stressor exposure results in increased permeability of mucosal epithelial are still being investigated.

Currently, there is good evidence to suggest that mast cells, corticotropin-releasing hormone (CRH), and neural activation are all critically involved in the increased permeability of the mucosal barrier to bacterial translocation during stressor exposure. Exactly how all these factors interact to result in mucosal barrier damage is unknown. One hypothesis is that exposure to a stressor results in activation of the sympathetic nervous system and the release of CRH from postganglionic neurons stimulates mast cell degranulation resulting in an inflammatory cascade and damage to epithelial cells (Soderholm and Perdue, 2001; Soderholm et al., 2002; Webster et al., 1998). Studies have shown that rats deficient in mast cells are protected from stress-induced barrier dysfunction and increased mucosal permeability (Castagliuolo et al., 1998; Santos et al., 2000). Mast cells are often found in close proximity to neurons (Heine and Forster, 1975; Stead et al., 1990) and administration of CRH to

laboratory animals results in mast cell degranulation (Theoharides et al., 1995) and increased colonic ion secretion and permeability similar to that observed in animals exposed to a laboratory stressor (Santos et al., 1999). Furthermore, blockade of CRH receptors or inhibiting adrenergic nerve excitability prevents stress-induced mucosal dysfunction and increased permeability (Gareau et al., 2006; Santos et al., 1999; Yang et al., 2006). The idea that CRH may initiate inflammatory responses during times of stress is not unique to the gastrointestinal tract, but has been proposed as a critical mediator in many autoimmune/inflammatory phenomena (Crofford et al., 1992; Kawahito et al., 1995; Webster et al., 1998).

The mechanism(s) for stress-induced bacterial translocation and endogenous LPS release, as well the nature of the potential eHsp72/LPS association remain a topic of current investigation. It is possible that the increase in LPS and eHsp72 concentrations in the blood after stress occurs via independent releasing mechanisms and from different cell types or tissues. If this were so, then the two independently released proteins would enter the circulation separately and associate with each other in the blood. This is possible given that Hsp72 has been reported to readily directly bind to LPS (Byrd et al., 1999; Reed et al., 2003; Triantafilou et al., 2001a, b, c). A second possibility is that eHsp72 directly participates in the changes in gut permeability, thus mechanistically contributing to bacterial translocation. There is evidence for this idea as well. Neal et al. (2006) reported that activation of TLR4 on enterocytes is both necessary and sufficient to induce both the phagocytosis and translocation of gram negative bacteria across the intestinal barrier. In addition, exposure to stressors has been shown to stimulate intestinal epithelia bacterial uptake (Velin et al., 2004). Thus, the early wave eHsp72 released into the blood could bind to TLR4 and initiate bacterial translocation, leading to the later elevation in LPS in the blood. The eHsp72/LPS complexes then could form in the blood after the intestinal enterocytes have been stimulated. A third possibility is that the enterocytes themselves are the cellular source of the eHsp72 released after stress and that LPS is complexed to Hsp72 within the enterocyte, prior to release. Thus the enterocytes release the eHsp72/LPS complex directly either via receptor mediated exocytosis or necrotic cell death. A final possibility is that the eHsp72 and LPS are derived from and released from independent sources, but that the eHsp72/LPS association occurs in the mesenteric lymph nodes. We have previously reported that tail shock stress rapidly elevates Hsp72 in the mesenteric lymph nodes (Campisi et al., 2003c; Fleshner et al., 2006; Nickerson et al., 2006). In addition, LPS concentrations also increase in the mesenteric lymph nodes after tail shock stress. Thus it is possible that activation of the intense stress response could trigger the up-regulation mesenteric lymph node eHsp72 and bacterial translocation. Consequently LPS could accumulates in the mesenteric lymph nodes where it comes in contact with high concentrations of eHsp72, binds the LPS, and the eHsp72/LPS complex is released into the blood.

We have previously reported that tailshock is a stressor that results in bacterial translocation as indicated by an increase in circulating endotoxin levels (Fleshner et al., 2007). In addition, we have shown that exposure to tailshock stress increases blood concentrations of both LPS and eHsp72, that these increases occur with similar

kinetics, and removal of LPS from plasma via Detoxi-Gel column reduced by ∼80% the increase in eHsp72 found in plasma from stressed, but not control, rats (Fleshner et al., 2007). These observations and the ideas previously presented, contributed to the development of our hypothesis that the gut may be one predominant tissue source of released eHsp72, and that NE released from sympathetic neurons innervating the small intestine (duodenum, jejunum and ileum), binds to α1-ADR expressed on gut epithelial cells and triggers rapid release of available cytosolic Hsp72 stores. If the intestine is an important tissue source for the rapidly release eHsp72 found in the blood after stressor exposure, then basal or resting concentrations of Hsp72 should be relatively high in the small intestine compared with other secretory tissues (Figure 2). If NE binding α1-ADR is an important releasing signal, then small intestine (specific mucosal layer) should be sympathetically innervated and α1-ADR should be expressed on gut epithelial cells. Both stipulations are supported by the literature (Baglole et al., 2007; Furness, 2000; Nasser et al., 2006; Phillips et al., 2006; See et al., 1990). Although still preliminary, we present data that support this hypothesis.

There is evidence that eHsp72 found in the blood after stressor exposure is derived from "the hepatic-splenic viscera" (Febbraio et al., 2002). Importantly, eHsp72 is released rapidly (10–20 min) after exposure to a systematic stressors such as exercise (Walsh et al., 2001) or tailshock stress (Campisi et al., 2003b). Thus it seems likely that the Hsp72 that is first released and detected in the blood is derived from existing cellular stores. In fact, Abboud et al. (2008) recently reported that the amount of eHsp72 released into the supernatant from THP cells in vitro did not change with addition of a protein synthesis inhibitor. We hypothesize that the small intestine is a likely tissue source of eHsp72. If this is correct, then small intestine compared with

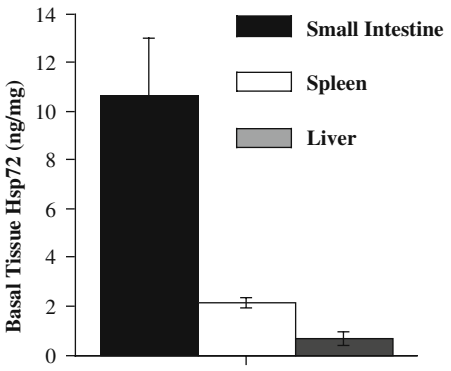

Figure 2. Small intestine has higher basal concentrations of Hsp72 than does spleen or liver. Control specific pathogen free, adult male Fischer 344 rats were sacrificed via rapid decapitation. Intestine (duodenum and jejunum), spleen and liver segements were dissected and flash frozen. Tissues were homogenized. Hsp72 concentration was measured using ELISA. Protein concentration was measured using the Bradford. Data are presented as ng of Hsp72 per mg of sample protein. Bars represent group means (n=5) ± SEM.

other "hepatic-splenic" tissues, should have relatively high concentrations of intracellular stores of Hsp72 prior to stressor exposure. To test this, adult male F344 rats (n=5) were sacrificed via rapid decapitation. Tissues were rapidly dissected, frozen in liquid nitrogen and homogenized for Hsp72 measurement (ELISA, Assay Design). Data are presented as Hsp72 ng per mg total protein (Bradford). As shown in Figure 2, small intestine (duodenum and jejunum) has 3–4 times higher Hsp72 concentration (ng/mg) in than in spleen or liver under non-stressed, resting or basal conditions. High concentrations of Hsp72 in gut may be due to chronic exposure to the endogenous bacterial flora (Arvans et al., 2005). Thus small intestine has large cytosolic "stores" of eHsp72 available for immediate release into the blood. Interestingly, the venous return from the small intestine enters mesenteric lymph nodes, spleen and liver, making it feasible that the stress-induced Hsp72/LPS complexes could rapidly gain access to these immune relevant tissues.

If the hypothesis that systemic stressors induce eHsp72 release from gut epithelial cells via α1-ADR is correct, then the following should also be true: (1) consistent with our previous reports (Johnson et al., 2005; Johnson and Fleshner, 2006), prazosin (α1-ADR antagonist) should block the rapid stress-induced eHsp72 release; (2) treatment with a selective intestinal Hsp72 co-inducer prior to stressor exposure, would be expected to increase stress-evoked eHsp72 increases in the blood; and (3) gut epithelial cells should release Hsp72 after direct α1-ADR stimulation. We conducted the following studies to test these ideas.

Adult male F344 rats (n=5 per group) served in one of the following groups: (1) no stress controls (Control), (2) exposure to 50, 5-s, 1.6 mA inescapable tailshocks (Stress, 50 IS) as previously described (Campisi and Fleshner, 2003; Campisi et al., 2003b; Johnson et al., 2005; Johnson and Fleshner, 2006), (3) treatment with an intraperitoneal injection of prazosin (2.0 mg/kg) 30 min prior to stressor exposure (Prazosin + IS), or (4) treated orally with 5-aminosalicylic acid (5-ASA, 50 mg/kg) for 4 days prior to stressor exposure. The choice of 5-ASA or mesalamine and oral dosing protocol was based on previously published work (Burress et al., 1997; Nandi et al., 2008). Rats were sacrificed via rapid decapitation immediately after stressor termination and trunk blood was collected on EDTA. Samples were quickly spun. Plasma was removed, aliquoted and frozen -80C for future assays. eHsp72 concentrations were measured using ELISA (Assay Design). Figure 3, clearly shows that stressor exposure once again increased blood concentrations of eHsp72 and prazosin blocked the increase. These data both replicate and extend our previous work and demonstrate that prazosin blocks the eHsp72 increase after both 50 (Figure 3) and 100 (Johnson et al., 2005) tailshocks. Animals pre-treated with 5-ASA had a ∼50% greater increase in plasma eHsp72 compared with the increase produced after 50 tailshocks alone. These data are consistent with our hypothesis.

The final study tested if gut epithelial cells would release Hsp72 after direct α1-ADR stimulation. Using an in vitro approach, IEC-6 cells derived from intestinal epithelial (ileum) cells (ATCC, CRL-1592, passage 21), were used. Cells were grown in Dulbecco′s modified Eagle medium containing 10% fetal bovine serum, 0.1 units/mL of pancreatic bovine, 1.5 g/L sodium carbonate, and 1.0 mL/100 mL of

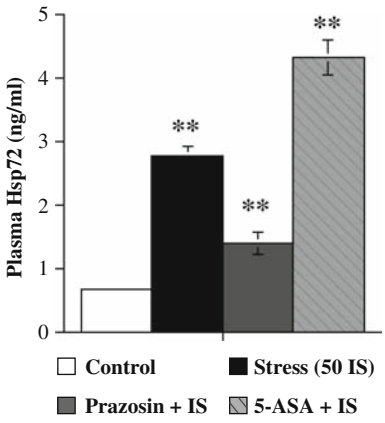

Figure 3. Exposure to tailshock stress (Stress, 50 IS) significantly increased circulating levels of extracellular Hsp72 (eHsp72) above non-stressed controls (Control). Prazosin (α1-ADR antagonist) blocked the tailshock-induced increase in eHsp72 (Prazosin + IS). Pretreatment with the Hsp72 co-inducer 5-aminosalicylic acid, doubled the tailshock-induced increase in eHsp72 (5-ASA + IS). Blood samples were collected via rapid decapitation and plasma Hsp72 was measured by ELISA. Bars represent group means (n=5) ± SEM. ∗∗denotes p<0.01 compared to Controls.

penicillin/streptomycin. Cells were cultured on three 6-well plates and immediately prior to experimental conditions the cells were fed fresh culture media. Because these cells are in vitro and are not exposed to endogenous bacterial flora that induces intracellular Hsp72, IEC-6 cells have low basal intracellular Hsp72 concentrations. To better mimic the in vivo Hsp72 concentrations, we heat shocked cells to increase intracellular Hsp72 levels in IEC cells. Specifically, two 6-well plates were heat shocked at 43C for 60 min in a water bath (Heat Stressed). Cells were then placed in a 37C, 5% CO_2 incubator for 24 h to recover. A third plate for basal measurement (Control) remained in the 37C, 5% CO_2 incubator. After recovery, the media was aspirated and the cells were treated with either 0, 0.5 or 1.0 mM phenylephrine (an α1-ADR agonist) in fresh culture media for 2 h. Following treatment, the supernatant was collected and spun at 150×g to remove debris and aliquoted for future assays. Hsp72 concentration in both the IEC-6 lysates and supernatants were determined via ELISA (Assay Designs). Assessment of necrotic cell death was determined by measuring lactate dehydrogenase in the supernatant (Promega, CytoTox 96 Assay). As shown in Figure 4a, intracellular Hsp72 levels are low in control IEC-6 cells. Heat shock but not phenylephrine, increases intracellular Hsp72 concentrations. Figure 4b reveals that phenylephrine dose dependently increased extracellular Hsp72 in heat stressed but not control cell supernatants. Figure 4c shows that although there is a slight increase in the percentage of dead cells after heat shock, phenylephrine did not increase cell death. Together these data support the hypothesis that Hsp72 can be released from gut epithelial cells by activating the α1-ADR and that this release is a receptor mediated and is independent of necrotic cell death.

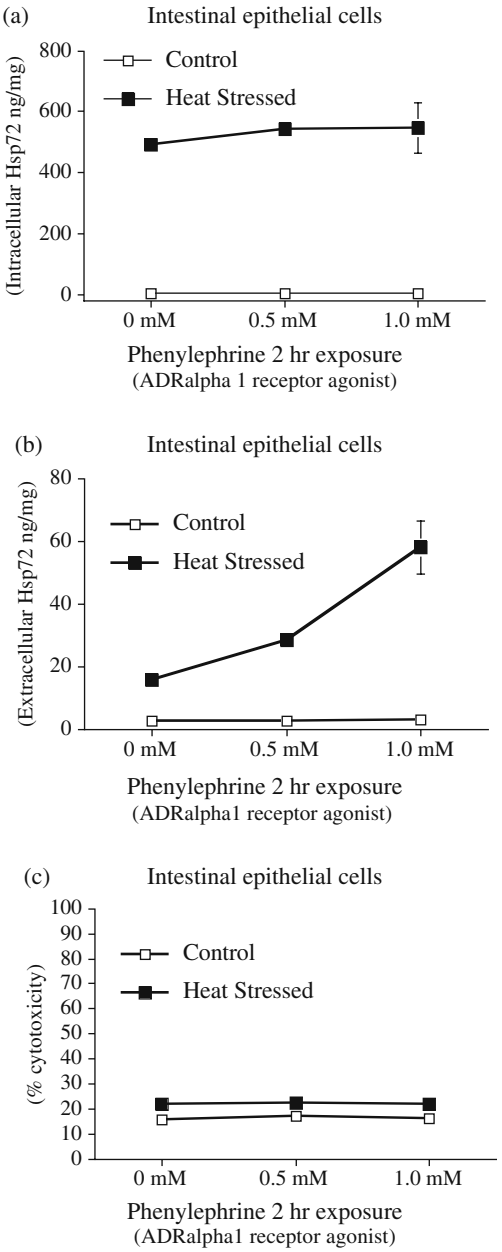

Figure 4. Heat shock increases intracellular Hsp72 concentration of IEC-6 cells. (**a**) Phenylephrine (α1-ADR agonist) induces the release of Hsp72 into the supernatant of heat shocked but not control IEC-6 cells, suggesting that Hsp72 release is triggered by α1-ADR (**b**). This release is not due to necrotic cell death (**C**).

CONCLUSION

Acute activation of the stress response modulates many aspects of physiology, including the immune system. There is convincing evidence that tissues release "alarm signals" in response to local and/or systemic challenge, and that these signals can initiate and direct inflammatory responses. The goal of this chapter was to develop the hypothesis that Hsp72 when released into the extracellular environment, can function as an alarm signal. Furthermore, we make the case that the nature of the immunological consequences of Hsp72 depends on the cellular distribution (i.e., intracellular, cell surface and/or extracellular) and the cellular source (e.g., tumors, viral infected cells, normal tissues: intestine, vasculature, macrophages, B cells, or neutrophils) and consequently, the type of peptides found in the Hsp72 substrate binding domain (e.g., tumor antigens, viral peptides, or LPS). We propose that activation of the systemic in vivo stress response releases eHsp72 from the small intestine and that the eHsp72 that is released is complexed to LPS derived from endogenous enteric bacteria. In vivo, this eHsp72/LPS complex is capable of modulating specifically inflammatory processes. If our hypothesis is correct, then it helps to explain why LPS-stripped recombinant Hsp72 protein no longer stimulates inflammatory cytokine production in vitro. Thus the immune modulatory effects of Hsp72 in vivo likely work in concert with other aspects of the stress response such as bacterial translocation, to further promote the pro-inflammatory effects of stress.

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CHAPTER 13

THE 70 kDa HEAT SHOCK PROTEIN FAMILY AND LEARNING

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Abstract: This chapter examines data supporting the view that heat shock proteins members of the 70 kDa family (HSP70) interfere with the plastic mechanisms underlying learning and memory. In the first part, we present evidence that rodents trained in tasks taxing specific forms of learning show increased levels of inducible (Hsp72) or constitutive (Hsc70) HSP in the brain regions mediating these learning systems. In the second part, we describe experiments in which exposure to a heat shock (also referred to as heat shock preconditioning) prevents the disruptive effect of amnestic agents on learning performance and synaptic plasticity, and interferes with the learning performance in different species. In the third part, we review data aimed at disentangling specific from non-specific learning effects of increased expression of HSP70 in the brain. We conclude that the experimental conditions in which HSP70 are expressed and the loci where this expression takes place suggest a role for these proteins in synaptic plasticity, learning, and memory

Keywords: 70 kDa Heat shock protein; heat shock; heat shock factors; learning; stress; motor activity

Abbreviations: AD, Alzheimer's disease; fEPSP, field excitatory post synaptic potential; HSF, heat shock factor; HSP, heat shock proteins; HD, Huntington's disease; HSP70, 70 kDa heat shock protein family; Hsc70 and Hsp73, constitutive 70 kDa HSP; Hsp70 and Hsp72, inducible 70 kDa HSP; *hsp72*, stress inducible Hsp72 gene; LTM, long-term memory; LTP, long-term potentiation; PD, Parkinson's disease; STM, short-term memory

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INTRODUCTION

From Donald Hebb's assumption in 1949 that learning is based on the modification of synaptic connections between neurons, it is now well demonstrated that learning requires the formation of new synapses and/or the stabilization of pre-existing ones. Experience-dependent changes in synaptic strength alter neuronal excitability and ultimately modify connectivity in widespread brain circuits thereby enabling memory storage and retrieval. Specifically, rewiring of brain circuits depends upon a variety of components active at the synapse including newly synthetized proteins, cytoskeletal adaptors and signaling molecules representing several transduction pathways. Among the key components of this machinery, members of the 70 kDa heat shock protein family (HSP70) are the object of a growing interest. HSP70 family consists of several proteins (Table 1) some of which, like the heat shock cognate 70 (Hsc70 also named Hsp73), are constitutively expressed while others, like the heat shock protein 70 (Hsp70 also named Hsp72) are inducible. It has been proposed that Hsp70 would be requested to amplificate the chaperone function carried out under normal conditions by its cognate Hsc70 (Hightower et al., 1994). These proteins are synthesized in response to a variety of insults including hyperthermia (Brown, 1990; Nowak et al., 1990), ischemia (Pettigrew et al., 2003; Mariucci et al., 2007), infarct (Hutter et al., 1996; Gray et al., 1999), lesions (Planas et al., 1997; Allen and Chase, 2001; Reynolds and Allen, 2003), and seizures (Lowenstein et al., 1990), or following less drastic metabolic changes as those produced by physical exercise (Campisi et al., 2003a, b; Whitham and Forbes, 2008) or psychophysiological stress (Fukudo et al., 1995, 1999). Their primary role consists in preventing or repairing cell injury via their participation in different biological pathways. For example, a role for both Hsc70 and Hsp70 in antagonising oxidative stress (Calabrese et al., 2002, 2004; Papp et al., 2003), reducing inflammation (Campisi and Fleshner, 2003; Van Molle et al., 2002), preventing apoptosis (Takayama et al., 2003; Terui et al., 2004), or promoting immune responses (Campisi et al., 2003a, b; Asea, 2006) has been reported. In addition, there is evidence of their participation in the synthesis, assembly and degradation of proteins, with a specific involvement in promoting translocation of newly synthesised proteins to their target sites as well as in preventing aggregation by maintaining their original state of folding (Feige and Polla, 1994; Welch, 1991; Papp et al., 2003). Indeed, the fact that both Hsc70 and Hsp70 are synthesized in most neuronal cells suggests that their expression might also be used as a marker for metabolic activity in brain areas engaged in cognitive processing.

Table 1. Major members of eukaryotic 70 kDa heat shock protein family

Name (synonyms)	Expression	Intracellular location
Hsc70 (Hsp73)	Constitutive	Cytosol nucleus
Hsp70 (Hsp72)	Inducible	Cytosol nucleus
BiP (GRP78)	Inducible	Endoplasmic reticulum
GRP75	Inducible	Mitochondria

However, whether these proteins participate in synaptic signalling at brain sites critically involved in the formation and the storage of memory traces is still a matter of debate. The argument we present is that both the experimental conditions in which HSP70 are expressed and the loci where this expression takes place make it possible they interfere with the molecular machinery governing synaptic plasticity, learning, and memory.

LEARNING-RELATED CHANGES IN 70 KDa HSP FAMILY

Evidence from human and experimental animal studies indicates that memory is organized into independent systems controlling distinct cognitive functions through selectively engaged neural circuits. Specifically, the notion of multiple brain memory systems arises from the observation that lesions to restricted anatomical regions abolish certain memory operations but leave other forms of acquisition intact (Scoville and Milner, 1957; Cohen and Squire, 1980). In rodents, for example, lesions to brain regions lying in the temporal lobe disrupt declarative, spatial or context-based memory but spare the capability to form stimulus-response associations or motor habits. On the other hand, damage circumscribed to the caudate putamen interferes with stimulus-response associations or motor habits without significantly affecting cognitive learning (Packard et al., 1989; Packard and White, 1991; McDonald and White, 1993). Neuroimaging data in humans also provides evidence in favor of independent neural systems mediating different types of memory (Poldrack et al., 2001). Thus, one basic requirement for identifying learning-associated proteins consists in choosing a task taxing specific memory operations and examining protein expression in regions belonging to the dedicated neural circuit.

HSP72 is Induced in the Cerebellum Following Active Avoidance Learning

Active avoidance learning is an example of stimulus-response association learning. In general, active avoidance in rodents is assessed in a two-compartment cage and the standard protocol consists in presenting a neutral stimulus (e.g. light) before the onset of an aversive unconditional stimulus (US: foot-shock). Following several light – shock presentations, the light is identified as a predictor of the aversive stimulus and can be used as a signal (conditioned stimulus: CS) for changing compartment before the foot-shock is delivered. Animals facing this situation can emit three types of responses: escape responses (changing compartment when the foot-shock is administered), conditioned responses (changing compartment during the CS presentation), and freezing responses (staying in the compartment where the shock is delivered). Extensive training in this task can elicit automatic avoidance responses (changing compartment at the very onset of the CS presentation) that show the properties

of motor-based routines. For example, these responses are sensitive to corticostriatal pathway alterations (Kelly et al., 1977), dopaminergic depletion (Mitcham and Thomas, 1972; Neill et al., 1974), and mutations affecting the cerebellum function as tenascin-R deficiency (Montag Sallaz and Montag, 2003) or the Lurcher mutation (Monfort et al., 1998). Based on these observations, we trained rats in the active avoidance task and measured the expression of the constitutive Hsp73 and the inducible Hsp72 immediately, 4, 8 or 20 h post-training in the neocortex, hippocampus, cerebellum and brainstem as well as in peripheral tissues (liver and kidney) with the prediction that the cerebellum would show enhanced Hsp72 expression. To control the learning-specificity of Hsp73 and Hsp72 expression, measurements were also carried out in control groups including foot-shocked rats, light-stimulated rats, control cage rats (negative controls), and hyperthermic rats (positive controls). Measurements of both Hsp72 and Hsp73 levels were performed by western blotting and analysed using a semi-quantitative method (Ambrosini et al., 1999). Behavioral data showed that the majority of the rats (80%) strongly increased their avoidance scores as training proceeded while some rats (15%) were low avoiders, and a few rats (5%) exhibited only freezing. Measurements of the constitutive Hsp73 revealed that this protein was expressed in all brain regions of the control rats but, also, in all brain regions of the trained rats, irrespective of their performance level or of the post-training interval at which measurements were performed. Conversely, Hsp72 expression was low in the cerebral hemispheres and the brainstem but highly expressed in the hippocampus and the cerebellum of the trained rats, with an increase in the induction rate occurring between immediate post-training and the 4 h time-point. However, induction of Hsp72 in the hippocampus was also found in control rats receiving only foot-shocks and in one light-stimulated rat. Thus, no correlation was evident between active avoidance scores and Hsp72 levels in the neocortex, hippocampus and brainstem while Hsp72 levels in the cerebellum were positively correlated with escape responses ($r = 0.67$, $P < 0.05$) (Figure 1a) at time-point 0 and, importantly, with avoidance responses ($r = 0.78$, $P < 0.01$) (Figure 1b) at the 8 h time-point. Thus, in agreement with findings showing a role for the cerebellum in motor-based and stimulus-response learning, we found intense Hsp72 expression in the cerebellum of rats showing initial high escape scores then high avoidance scores thereby suggesting that cerebellar Hsp72 is synthesized in relation to distinct, time-specific, cerebellum-mediated operations. Specifically, we hypothesized that the former correlation may reflect an initial involvement of the cerebellum in foot-shock-elicited motor responding while the latter correlation may underlie the storage of a novel motor program implemented on the basis of CS-US associations.

In relation to these findings, although no correlations were detected between active avoidance scores and Hsp72 levels in liver and kidney, we found that Hsp72 expression was high in the liver of trained rats immediately post-training. Assuming that Hsp72 may play a role in training-related adrenaline-dependent release of glucose otherwise shown to promote memory (Gold et al., 1986), this process could increase the salience of the CS and facilitate the shift from escape to avoidance responding.

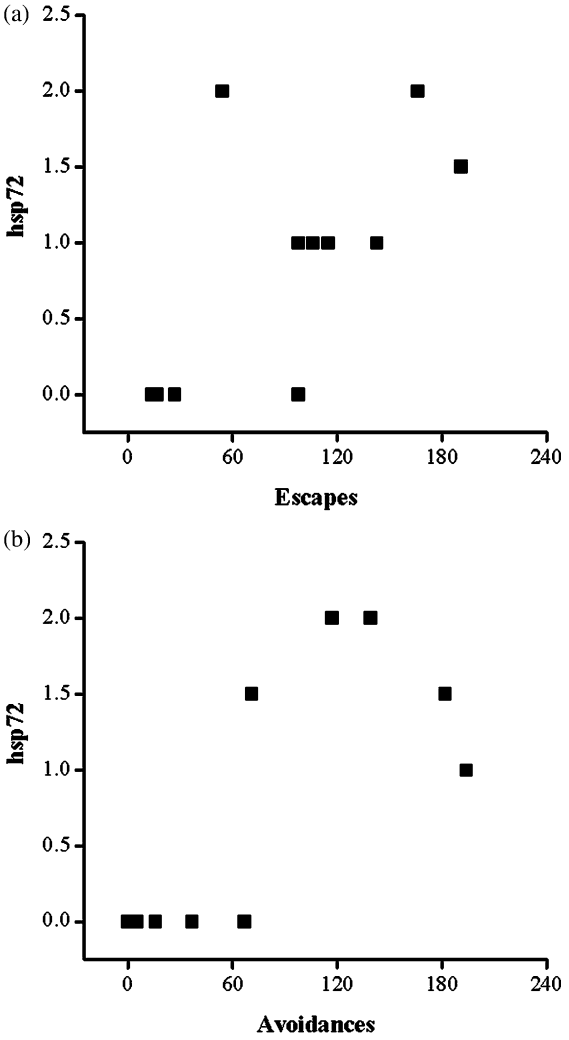


Figure 1. Scatterplot showing a positive correlation between number of escape responses and expression levels of Hsp72 ($r = 0.67$, $P < 0.05$) immediately following active avoidance training (**a**) and between number of avoidance responses and expression levels of Hsp72 ($r = 0.78$, $P < 0.01$) 8 h following active avoidance training (**b**) in rats cerebellum. Values of Hsp72 are expressed as arbitrary units.

HSP72 is Induced in the Hippocampus Following Inhibitory Avoidance Learning

Inhibitory avoidance is a learning task requiring the animal to associate an aversive experience with the context in which this experience occurs. This task has been extensively used to study memory consolidation in rodents since a variety of

treatments administered at different post-training intervals have revealed that memory can be enhanced or impaired in a time-dependent manner (see McGaugh and Izquierdo, 2000 for a review). The standard protocol consists in placing animals in a well-illuminated compartment connected by a sliding door to an adjacent dark compartment. Because of their photophobia, animals quickly enter the dark compartment where a mild foot-shock is delivered; they then are returned to their home cage. Clearly, memory of this aversive experience will make animals avoiding the dark compartment on subsequent returns to the apparatus. Avoidance behavior therefore depends on the capability to implement a hippocampal-dependent contextual representation and, hence, is disrupted by manipulations that decrease hippocampal functionality. Those include lesions (Cogan and Reeves, 1979), temporal inactivation (Lorenzini et al., 1996), and intra-hippocampal injections of substances affecting specific receptors, enzymes, or protein synthesis (see McGaugh and Izquierdo, 2000 for a review). Importantly, data showing that rats trained for inhibitory avoidance display an increase in the amplitude of evoked synaptic transmission in vivo in CA1 and exhibit changes in hippocampal glutamate receptors similar to those induced by high-frequency stimulation of the CA3-CA1 pathway (Whitlock et al., 2006) further demonstrate that this form of conditioning elicits robust plastic changes in hippocampal circuits. Based on these findings, we trained rats for inhibitory active avoidance and measured Hsp72 expression by western blot in the cerebral cortex, hippocampus, and cerebellum following testing with the hypothesis that significant changes in protein expression would be maximally found in the hippocampus (Mariucci et al., in preparation). Training and testing were run on the same day using a protocol in which two training sessions and one testing session were administered at 120-s intervals. There is, in fact, evidence that rats shocked in the dark compartment have a propensity to re-enter it if returned to the apparatus shortly after the shock but start showing inhibitory avoidance on subsequent returns. The advantage of this protocol is to compact training and testing in order to measure Hsp72 levels as soon as the foot-shock-context association is established. In this experiment, the intensity of the foot-shock delivered during the training trials was fixed at 0.6 mA and lasted 5 s and the variable estimating memory was the difference in time to enter the dark compartment (response latency) between training and testing. Induction of Hsp72 was investigated either 4 or 8 h following testing and in control groups including non shocked rats allowed to explore the apparatus for 120 s (active controls), rats that were shocked in a different chamber (shocked controls), and rats left in their home cage (naïve). Levels of Hsp72 were determined by western blotting, quantified by using an image analyzer (Image-Pro Plus, Media Cybernetics, Silver Spring, MD; USA) and expressed as optical density (OD) unit. During training, no difference in the latency to enter the dark compartment was found between trained animals and active controls while, during testing, latency consistently increased in the trained animals (Figure 2a). Examination of Hsp72 levels revealed that the peak of expression was in the hippocampus of trained rats sacrificed 4 h following testing (Figuer 2b). Specifically, a one-way ANOVA showed a main effect of the group

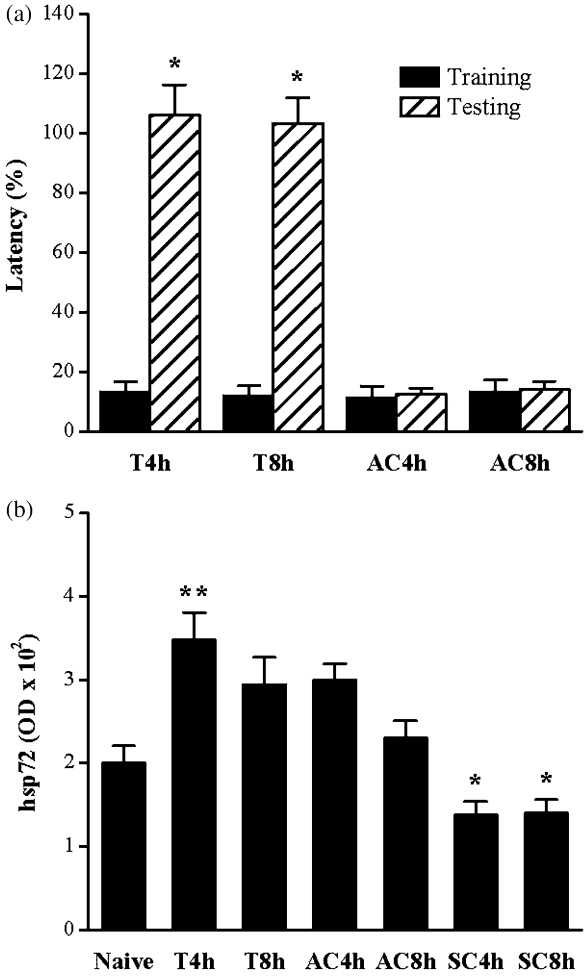


Figure 2. Response latencies (Mean ± SEM) during passive avoidance training and testing. $^{*}P < 0.001$ Training vs Testing, Student's *t*-test for paired samples (**a**). Hippocampal Hsp72 expression (Mean ± SEM) in control cage (naïve), trained (T), active controls (AC), and only shocked (SC) rats measured 4 or 8 h following passive avoidance testing (**b**). $^{*}P < 0.01$ vs T4h, T8h and AC 4h; $^{**}P < 0.01$ vs Naïve (Bonferroni test).

factor with post-hoc pair comparisons indicating that hps72 expression in the hippocampus was significantly higher 4 h following testing than in the hippocampus of naïve or only shocked rats ($P < 0.01$ for all comparisons). Interestingly, lower levels of Hsp72 expression were found in active controls examined at the same time point (4 h) following exploration of the apparatus, or in trained rats 8 h following testing. Finally, no significant variation of Hsp72 levels between trained and control groups

was found in the cortex or the cerebellum. The observation that Hsp72 levels in trained animals were decreased between 4 and 8 h are, therefore, consistent with data showing that treatments affecting memory consolidation are effective when administered at about 6 h of acquisition, that is, during the time-window protein synthesis is thought to take place. Thus, although additional experiments measuring hippocampal Hsp72 levels at other time-points are necessary to confirm the existence of a gradient, these preliminary observations lend support to the view that it is not the shock per se but the implementation of an adaptive inhibitory response that promotes gradual expression of Hsp72 in the hippocampus.

Water-Maze Training Enhances Expression of the Constitutive HSC70 in the Hippocampus

The involvement of HSP70 family in learning was assessed in rats subjected to spatial learning in the Morris water maze (MWM), that is, a task taxing hippocampal-dependent learning (Pizarro et al., 2003). The aim of this experiment was still to dissociate a learning from a stress effect on HSP induction, and the protein examined was the Hsc70, the constitutive HSP of the 70 kDa family. This protein shows three interesting properties. First, it is localized at the synapses and mostly at post-synaptic rather than pre-synaptic sites (Hu et al., 1998; Suzuki et al., 1999). Second, there is evidence that the *Hsc70* gene is expressed during synaptic plasticity (Nevidi et al., 1992). Third, Hsc70 levels correlate with the cell metabolic activity (Welch, 1993) raising the possibility that, independently from a specific role in synaptic plasticity, enhanced levels of Hsc70 could be used as markers for increased cellular metabolism associated with the learning experience. In these experiments, five groups of rats and their yoked controls were trained for different durations (1, 2, 3, 4, or 5 days) in the MWM. This task requires rats to swim in a pool filled with opaque water until a hidden safety platform situated below the surface of the water is discovered. The latency to find the platform, which is expected to decrease across trials, estimates the learning performance. Following training, the platform is removed and the time rats spent swimming in the quadrant of the pool where the platform was previously located measures the retention performance. It is well known that the platform location in the MWM can be identified on the basis of two distinct orientation modalities. One is egocentric orientation when, for example, an explicit stimulus suspended above the platform marks its position and rats just have to swim in the direction of the stimulus. Egocentric orientation is hippocampal-independent but requires an intact dorsolateral striatum (Packard and McGaugh, 1992). The other one is allocentric orientation that consists in inferring the platform location from triangulation among stimuli located around the pool. Implementation of allocentric orientation requires an intact hippocampus (O'Keefe and Nadel, 1978) and the present MWM protocol was designed in such a way that rats had to rely on in this modality. Although the training duration varied between groups, all rats were sacrificed 24 h following the last training trial and their brains were processed to measure Hsc70 mRNA in the

CA1, CA3 and dentate gyrus (DG) regions, and to globally determine Hsc70 protein levels in the dorsal hippocampus. Results showed that rats trained for 2 or 3 days exhibited both a significant increase in mRNA expression of Hsc70 in DG, CA1 and CA3, and of Hsc70 protein levels in the dorsal hippocampus. Interestingly, the observation that no change in Hsc70 expression occurred in yoked rats that swam for 2 or 3 days without the possibility of finding the safety platform ruled out that the enhancement found in the trained rats was stress-related. Remarkably, no change in Hsc70 expression emerged either at the very first exposure to the task (day 1) or when the task was well-mastered (day 4 and 5). These observations are in good agreement with data showing that c-*fos* mRNA expression in hippocampal subfields follows a different time course during distinct phases of acquisition and recall of a bar-press operant discriminative task (Bertaina-Anglade et al., 2000). Specifically, no increase in hippcoampal c-fos activity was detected either at the beginning of training, i.e., when rats basically process sensorial information, or at the end of training, i.e., when the task is well-mastered and no longer solicits hippocampal-based operations. Thus, increased expression of Hsc70 in the hippocampus on days 2 and 3 is likely to be related to implementation of efficient patrolling in the MWM and therefore argues in favor of a learning-related effect. Nevertheless, paradoxical findings like the observation that mRNA expression in all hippocampal regions was increased in the yoked rats on day 3 despite the fact that these animals never experienced an explicit learning rule challenge the specific role of this HSP in the learning process.

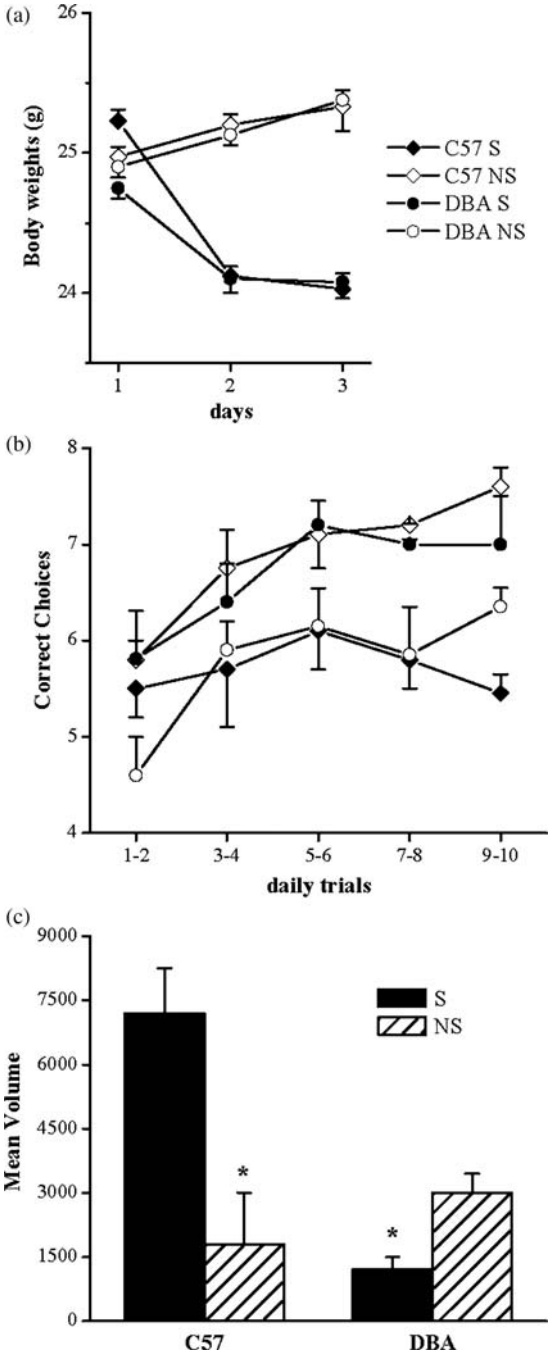
Radial Maze Learning Induces HSP72 Expression in the Hippocampus Independently from Exposure to Stress

One possibility to uncouple stress and learning performance is provided by inbred mice that show both differences in hippocampal-dependent learning (Ammassari-Teule and Caprioli, 1985; Upchurch and Wehner, 1989; Ammassari-Teule et al., 2000) together with an opposite stress-induced modulation of learning. For example, retention of passive avoidance following post-training immobilisation stress is impaired in DBA/2 J (DBA) mice but enhanced in C57/BL6J (C57) mice (Castellano and Puglisi-Allegra, 1983). Interestingly, in the same task, post-trial corticosterone injections that directly modulate the activity of the hypothalamic-pituitary-adrenal axis also affect retention performance of each strain in an opposite fashion (Cabib et al., 1996). Thus, examining learning-associated expression of HSP in C57 and DBA mice exposed to a stressful experience would permit to clarify whether induction of these proteins is maximal in stressed animals irrespective their perform bad or well, or in high or low performing animals irrespective they are or they are not exposed to stress. A point to be considered is that the active avoidance and MWM tasks used so far for assessing learning-induced changes in HSP expression involve aversive stimuli whose manipulation directly affects the rate of acquisition (Archer, 1982; Akirav et al., 2001). These protocols make it a priori difficult determining

whether increased HSP expression is driven by task-specific stressful stimuli, reflects learning-associated cellular changes, or both. To circumvent this difficulty, we chose to analyse the effect of stress (acoustic stress) on C57 and DBA learning performance using a radial maze, a positively reinforced hipppocampal-dependent spatial task (Ambrosini et al., 2005). Exposure to acoustic stress started two days before pre-training and lasted until the completion of the behavioral experiments. Stressed mice were placed in a cage inserted in soundproof cubicle and exposed to a broad band 80 dB noise delivered 5 h per day via a loudspeaker situated above the cages. Training began 90 min after the noise was interrupted. Mice ran the maze according to a Latin square design applied during the 10 days of training to balance individual differences in the time elapsing between the end of acoutic stress and the training trial. Half of each mouse strain was exposed to acoustic stress for a total period of 16 days whereas the other half was left undisturbed in the animal house. These two groups were then divided into three subgroups: food deprived mice subjected to radial maze training, food deprived active control mice, i.e., mice placed in the maze for a time equivalent to the mean of the time spent there by the trained group and allowed to eat food scattered along the arms, passive controls, i.e., non trained control mice. Training was carried out in a radial eight maze with each arm baited with a food pellet.

At the beginning of the trial, mice were placed on the central platform of the maze and allowed to make eight runs. An errorless trial consisted in visiting each arm only once and collecting the eight food pellets. Returns to previously visited arms were considered as errors. Mice ran one trial per day during 10 consecutive days and their performance was estimated using both the time spent to complete each trial and the number of correct choices made during each trial. All mice were sacrificed 4 h following the last training trial, their hippocampi were dissected and then processed to measure Hsp72 levels by western blotting. Band intensities were compared by optical densitometry. First of all, a point to be noted is that the physiological effects of acoustic stress were similar in both strains as shown by the body weight curves that reveal a similar weight loss in stressed C57 and DBA (Figuer 3a). Also, the behavioral data confirmed the superiority of non stressed C57 mice, compared to non stressed DBA mice, in learning the radial maze task. Extensive evidence indicates that this difference in performance is due to genetic variations in hippocampal morphology and plasticity with C57 showing the most functional hippocampus. Interestingly, acoustic stress also induced a strain-specific

Figure 3. Mean (± SEM) body weights (in g) of C57 and DBA mice non exposed (NS) or exposed (S) to chronic acoustic stress. The values correspond to measurements performed from the day preceding the first exposure to the noise (D1) until the day preceding pre-training (D3) (**a**). Number (Mean ± SEM) of correct choices made on each daily trial during radial maze training in C57 and DBA mice non exposed (NS) or exposed (S) to chronic acoustic stress (**b**) Densitometric analysis (Mean ± SEM) of Hsp72 expression in the hippocampal tissue of trained mice (**c**). *NS *vs* S mice, $P < 0.05$.


(a)
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C57 S
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Figure 3.

effect on learning since stressed C57 mice performed worse and stressed DBA mice performed better than their non stressed counterpart (Figure 3b). Most remarkably, post-training expression of hippocampal Hsp72 in the various strain x stress conditions was found to correlate with radial maze performance rather than with the stressful experience per se. Specifically, post-training expression of Hsp72 was low in the condition in which each strain showed the highest performance – non stressed C57 and stressed DBA – and high in the condition in which each strain showed the lowest performance– stressed C57 and non stressed DBA (Figure 3c). A point to be noted is that the physiological effects of acoustic stress were similar in both strains as shown by the body weight curves that reveal a similar weight loss in stressed C57 and DBA. On the one hand, the finding that Hsp72 was scarcely expressed in the hippocampus of high spatial learners is consistent with the Pizarro et al. (2003) data indicating that hippocampal Hsc70 expression measured in rats following MWM training strongly decreased after the task was mastered. Thus, as already observed for other learning-associated proteins, like FOS (Anokhin and Rose, 1990; Bertaina-Anglade et al., 2000; Nikolaev et al., 1992) ZIF and JUN (Grimm and Tiechmeyer, 1997) and ARC (Kelly and Deadwyler, 2002), post-training expression of HSP in the hippocampus is down-regulated after the performance is stabilized. On the other hand, there is an apparent discrepancy between the Pizarro et al. (2003) observation that Hsc70 is maximally expressed when rats were starting to master the task, and our finding that hippocampal Hsp72 is strongly expressed by the end of training in poor spatial learners. It is unlikely that, in our experiment, poor-learner mice were discovering the experimental rule at the time training was stopped since no improvement in performance was evident from trial 5 to trial 10 in stressed C57 and non stressed DBA. It is therefore apparent that the time course of HSP expression and, overall, its relation to performance are critical factors for elucidating the role played by these proteins in learning-associated cellular changes. For example, it could be that a peak of HSP expression in the hippocampus occurring when performance is increasing is an index that the task is being mastered and new neural connections are being established (Pizarro et al., 2003). Conversely, a sustained expression of HPSs in the hippocampus of mice that show persisting problem-solving difficulties might simply reflect an increase in metabolic activity related to the effortful engagement of the hippocampus in spatial information processing.

HEAT SHOCK-INDUCED CHANGES IN LEARNING

As an alternative to studies based on learning-induced HSP expression the relationship between HSP and learning has also been investigated by exposing subjects to a heat shock (also referred to as heat shock preconditioning) and then assessing how this treatment affects their learning performance. In general, these studies include a pilot experiment in which the time course of heat shock-related protein induction is determined in order that the learning test is administered at the maximal HSP expression time-point.

Heat Shock Prevents Scopolamine-Induced Amnesia

Indirect support to a role for Hsp72 in learning and memory comes from data showing that scopolamine-induced amnesia in rats can be prevented by heat shock pretreatment (Hung et al., 2004). In this experiment, rats were kept under general anesthesia at 42°C $\pm$ 0.05 for 15 min and Hsp72 levels were measured by western blot in the frontal cortex, striatum, hippocampus and hypothalamus immediately, 16 or 48 h after the heat shock. Examination of the time course of Hsp72 induction revealed an increased expression of this protein in all brain regions only at the 16 h time-point. Subsequently, rats subjected to heat shock and control rats were injected with the cholinergic antagonist scopolamine before being trained or tested for inhibitory avoidance. In the present experiment, heat-shocked and non heat-shocked rats received scopolamine injections (1 mg/kg, i.p.) either 30 min before training or 30 min before the test. Passive avoidance conditioning was estimated by using their latency to enter the dark comportment during testing. Results showed that rats exposed to the heat shock 16 h before they received scopolamine injections were not amnestic contrary to rats that were heat-shocked only 48 h before they were injected with scopolamine, or to non heat-shocked rats. Conversely, when the heat shock was administered after scopolamine injections, rats tested 16 h later were amnestic. Thus, increased levels of Hsp72 at the time scopolamine was administered were effective in preventing amnesia but ineffective in reversing amnesia. The mechanisms involved in this effect are still unknown. Given that scopolamine-induced amnesia is, at least partly, mediated by a blockade of hippocampal muscarinic receptors, it could be that increased levels of Hsp72 in this region interfere with acetylcholine receptor binding. An alternative hypothesis suggested by the authors is that the heat shock enhances expression of a variety of proteins and, among them, proteins mediating hippocampal synaptic events that would compensate for the blockade of cholinergic muscarinic receptors. Data supporting this view are described hereafter.

Heat Shock Prevents Scopolamine-Induced Disruption of Long-Term Potentiation

Long-term potentiation (LTP) consists of a long-lasting increase in synaptic strength between two neurons resulting from high frequency stimulation of the presynaptic neuron. Extensive evidence indicates that LTP shares common properties with learning and memory (see Morris et al., 2003 for a review) suggesting it could be the cellular substrate mediating encoding and storage of information in the brain. In an attempt to precise the relationship between HSP and LTP, Lin et al. (2004) examined the possibility that heat shock-related induction of HSP (Hsp72) interferes with the development of hippocampal LTP in basal conditions or after it is disrupted by scopolamine injections. As in the experiment described above, a pilot study was conducted to identify the time course of heat shock induced expression of Hsp72. Three groups of rats were used in this study: one group was anesthetized and heat-shocked

according to the Hung et al. protocol (15-min exposure to 42°C), one group was only anesthetized (control), and one group was non anesthetized (naïve), this latter group enabling basal Hsp72 levels to be estimated. Results showed that Hsp72 expression, measured by immunohistochemistry, was maximal at the same post heat-shock interval (16 h) as in the Hung et al. (2004) experiment. This time-point was therefore chosen for performing the first set of LTP experiments. Accordingly, hippocampal slices were taken from heat-shocked, control and naïve rats and then processed to determine Hsp72 levels or to record field excitatory post synaptic potential (fEPSP) at the CA3-CA1 synapse. Immunohistochemical data first confirmed that, 16 h following the heat shock, rats showed higher Hsp72 expression in the CA1 hippocampal region than control rats which, in turn, showed higher Hsp72 expression than naïve rats. However, in vitro LTP induced by tetanic stimulation (3 trains of 100 pulses at 100 Hz separated by 30 s intervals) was not found to differ in intensity or duration between heat-shocked and control rats, thus ruling out the option that increased Hsp72 levels directly modulate LTP. Nevertheless, scopolamine added to the perfusion bath abolished LTP in the control rats but did not in the heat-shocked ones. The preventive effect of the heat shock on LTP disruption was, however, not found when the experiment was repeated at the 0 or 48 h time-points, indicating that neuroprotection requires maximal levels of Hsp72 induction. Altogether, these observations establish that increased levels of Hsp72 are effective in counteracting treatments disrupting both learning performance (see above) and learning-related cellular mechanisms but do not interfere with learning or LTP per se. Consistent with the neuroprotection hypothesis, heat shock-related Hsp72 induction has been shown to prevent glutamate-induced neuronal damage in the CA1, CA3 and dentate gyrus of rat hippocampal slices (Sato and Matsuki, 2002) even though, as previously underlined, the mechanism by which the HSP exert a neuroprotective effect is unknown. Brown (2007) proposed it could depend on a complex formed by costitutive Hsc70 and hyperthermia-induced Hsp40 in synapse enriched brain areas that would refold denatured proteins. A role for HSP from adjacent glial cells enhancing stress tolerance of neurons has also been evoked. Alternatively, as previously mentioned in the discussion of the Hung et al. experiment, considering that Hsp72 induction activates protein kinase C and mitogen-activated protein kinase, required for learning and LTP, it could be that more than producing neuroprotective effect, heat shock preconditioning overall compensates for the disruptive effect scopolamine by enhancing activation of learning-dependent signaling pathways.

Heat Shock Disrupts Habituation in Caenorhabditis Elegans

The data so far presented were obtained in rodents facing learning situations which implicate the association of one stimulus – or a configuration of stimulus (context) – with an explicit reinforcement. Other situations like, for example, sensitization and habituation, are considered as non-associative forms of learning as they only require subjects to adapt their response to a single stimulus they are repeatedly

exposed to. Specifically, if the chosen stimulus elicits a strong sensorial reaction, subjects become increasingly sensitive to it and show enhanced responding on subsequent exposure. This phenomenon is called sensitization. Alternatively, if the stimulus elicits only a mild sensorial reaction, subjects habituate to it and show decreased responding. The latter phenomenon, called habituation, is considered as highly adaptive as it basically permits irrelevant stimulus to be ignored in order to better focus on stimuli that are important for survival. Habituation is well conserved across phylogeny as it develops in many organisms including humans, mammals, birds, fishes, insects, and invertebrates. *C. elegans* is a worm whose nervous system comprises 302 neurons and its entire genome has been mapped and sequenced. This worm reacts to mechanical stimulus applied to the side of a Petri dish by swimming backwards, and there is evidence that this response can habituate since the distance, traveled during tap-withdrawal, tends to diminish when taps are repeatedly applied to the Petri dish (Rankin et al., 1990). The observation that short term habituation develops when single taps are delivered with short inter-stimuli intervals (ISI) while long term habituation requires stronger stimuli, i.e. a train of taps, suggests that *C. elegans* can show distinct forms of habituation possibly involving different cellular mechanisms. In an attempt to identify the role of GLR-1, an AMPA receptor homolog, in long term habituation, Rose et al. (2003) used green fluorescent constructs to visualize GLR-1 receptors in the mechanosensory circuits, and to mark the protein synaptobrevin in sensory neurons where it is selectively expressed. Both wild-type and GLR-1 knockout worms were used in this experiment. Data showed that the wild-type worm trained for long term habituation (trains of taps) and then examined 24 h later for long term memory (LTM), exhibited less GLR-1 expression than untrained animals. The fact that sinaptobrevin levels in sensory neurons were unchanged indicated that GLR-1 modifications were selectively post-synaptic. Interestingly, worms lacking GRL-1 did not show LTM. Successively, wild-type worms were exposed to a heat shock between training blocks in order to disrupt their habituation performance. This presumption was based on an interesting property of the heat shock that, when administered, makes stress protein synthesis overwhelming any ongoing protein synthesis, including that required for LTM. Exposing worms to heat shock was carried out by submerging Petri dishes sealed with paraffin in a water bath at 32°C for 40 min during the resting periods between training blocks. Results showed that the heat shock treatment resulted in a total blockade of the behavioral expression of long term memory and of changes in GLR-1 expression. In a subsequent experiment aimed at investigating the role of GLR-1 in memory reconsolidation, Rose and Rankin (2006) exposed previously trained worms to heat shock immediately after the presentation of a reminder (one tap). While non heat shocked worms showed intact recall and memory reconsolidation, heat shocked worms did not, thus demonstrating that HSP induction interferes with transduction pathways mediating reconsolidation. Therefore, despite the fact that these studies do not clarify which family the proteins induced by heat shock pretreatment belong to, they, however, point to an aspect needing careful consideration when studying the role of HSP in learning and memory. It is, in fact, crucial to keep in mind that, according

to the experimental protocol, HSP induction guides synthesis of plasticity-related proteins but, also, can act as a blocker of protein synthesis required for LTM and learning.

HSP EXPRESSION IN THE BRAIN: SPECIFIC VERSUS NON SPECIFIC LEARNING EFFECTS

Stress, Motor Activity, Learning and Hsp72 Expression: How to Disentangle Specific from Non-Specific Learning Effects

70 kDa proteins, especially Hsp72, are ubiquitously expressed following motor activity. However, whether this expression represents an index of motor learning or, alternatively, is the consequence of the fatigue (stress) resulting from intense physical exercise is equivocal. For example, there is evidence that rats subjected to motor learning in a treadmill show increased expression of myocardial Hsp72 and of several markers of systemic stress like increased serum corticosterone levels and decreased body weights (Brown et al., 2007). Nevertheless, physical activity has been paradoxically shown to rescue stress-induced cellular alterations (Dishman et al., 1995; Fleshner et al., 2003) and to prevent stress-related mood disorders (Bjornebekk et al., 2005). To delineate the role played by Hsp72 in these interconnected processes, Campisi et al. (2003a, b) examined how physical activity interferes with the response to stress. In their experiments, rats were reared in a running or an immobile wheel for 8 weeks before being exposed to stressors consisting of inescapable tail-shocks or exhaustive exercise. Activation of the stress response following exposure to the stressors was verified by measuring serum corticosterone while Hsp72 induction was assessed in brain and peripheral tissues. Results showed that serum corticosterone was elevated following exposure to each stressor in both active and sedentary rats, thus demonstrating that different pre-stress activity levels do not affect the amplitude of the stress response. However, despite their similar corticosterone levels, active but not sedentary rats showed an increase in Hsp72 expression in the dorsal vagal complex, frontal cortex and hippocampus as well as in peripheral tissues including the pituitary, adrenal, liver, spleen, mesenteric lymph nodes, and heart. These findings, therefore, indicate that pre-stress physical activity promotes greater and faster Hsp72 induction in response to stress. Given that corticosterone levels estimating the stress response per se were similar in active and sedentary rats, can greater and quicker Hsp72 induction found in the active rats be an index of their stronger resistance to stress enabling them, for example, to show less stress-induced learning deficits? This point was investigated by the same group in a successive study examining whether different duration of pre-test physical exercise prevent shuttle-box escape deficits in rats subjected to inescapable foot-shocks (Greenwood et al., 2007). It is widely accepted that escape deficit following inescapable foot-shocks is a form of learned helplessness, i.e., an index of depression in rodents. Results indicated

that rats reared in the running wheel for 6 weeks, but not 2 weeks, showed consistent reduction of conditioned freezing in response to unescapable shocks and did not show escape deficits. Several hypotheses can account for these findings. First, based on data showing that physical activity prevents mood disorders, active rats might be, as hypothesized above, less sensitive to stressful inescapable foot-shocks. Second, in terms of pure motor behavior, it could be that rats long used to move in a running wheel are less prompt to develop conditioned immobility. Indeed, data showing that motor activity produces hippocampal neurogenesis (Farmer et al., 2004; Naylor et al., 2005; Uda et al., 2006), which is beneficial for both memory (Wojtowicz et al., 2008) and depression (Sahay et al., 2007), do not help in identifying the primary mechanism responsible for enhanced Hsp72 expression in the active animals. Thus, even though a clear adaptive function of increased Hsp72 in frontal cortex and hippocampus emerges in the active rats, the wide range of interconnected processes potentially interfering with Hsp72 induction underlines the difficulty of attributing a specific function to these proteins in the absence of concomitant selective markers of stress, learning or depression.

70 kDa HSP and Learning-Associated Changes in Synaptic Activity

Considering the role of 70 kDa HSP in relation to the dual model of Short-Term Memory (STM) and Long-Term-memory (LTM), it is apparent that increased activity of Hsp73 is likely sufficient to chaperone post-translational modifications of pre-existent proteins required for STM while induction of Hsp72 might be mandatory to chaperone the synthesis and maturation of proteins required for LTM. Consistent with this idea, there is evidence that long-term sensitization of aplysia neurons requires increased synthesis of BiP (Kuhl et al., 1992), a member of the HSP70 family which transiently associates with nascent proteins emerging into the endoplasmic reticulum prior to achieving their final folded state (Pelham, 1989). As BiP expression is increased 3 h after the onset of sensitization, i.e., at the time of maximal protein synthesis, BiP likely contributes to protein changes essential for the formation of new presynaptic terminals. Furthermore, indirect support to the idea that Hsp73 and Hsp72 might have a role in synaptic activity is provided by the correlation existing between development of visual imprinting, an early postnatal form of learning mediating filial attachment in chicks, and increased level of clathrin heavy chain in the brain region (intermediate and medial part of the mesopallium: IMM) underlying recognition memory in this species (Solomonia et al., 1997). Clathrin-coated vesicles are known to be involved in receptor-mediated transport. In neurons, the main role of clathrin is to contribute to the recycling of synaptic vesicles in nerve terminals (Maycox et al., 1992). Synaptic recycling occurs at a faster rate than endocytosis in other cells, and is promoted both by the neuron-specific clathrin-binding protein AP180, which enhances clathrin assembly, and by the neuron-specific auxilin which stimulates ATP-dependent uncoating catalyzed by Hsp73 (Morgan et al.,

2001). These data suggest that the amount of brain clathrin increases in concomitance with the rate of recycling and uncoating of synaptic vesicles. Hence, it could be that induction of brain HSP70 elicited by visual imprinting might be triggered by the enhanced synaptic activity underlying memory storage in IMM. Conspicuous evidence for the involvement of HSP70 in learning-induced synaptic changes also comes from the observation that both constitutive and inducible forms are present at cerebral cortex and hippocampal synapses (Moon et al., 2001). In the dendritic compartment of early differentiated neurons, coupling anatomical and immunoreactivity methods has revealed that Hsp70, but not Hsc70, mostly co-localizes with PSD-75, a protein present in the Post Synaptic Density (PSD) area that forms the active zone of the dendritic spines. Because PSD contains a variety of components, including receptors, cytoskeletal adaptors and signalling molecules involved in the maintenance and the plasticity of synapses, the most plausible interpretation of their presence at synaptic sites in developing neurons would be that they are involved in the guidance of morphological development. According to this scheme, their contribution to the folding of structural proteins would be crucial to make filopodia-like protrusions evolving in mature dendritic spines. Translating this "holding and folding" activity to adult neurons, the participation of Hsp70 in local synthesis of structural proteins would enable remodelling of existing synapses and the formation of new synaptic contacts. These observations have led to the proposal that Hsp70 might significantly contribute to synaptic tagging (Frey and Morris, 1998), the process by which plasticity proteins are stably produced and released in selective learning-activated circuits. The finding that LTP is enhanced in mice overexpressing HSP70 is in line with this hypothesis (Ammon-Treiber et al., 2008).

Dysregulation of HSP70 Expression is Associated with Cognitive Diseases

Additional support for a role of HSP70 in learning comes from data showing that dysregulation of their expression is frequently associated with cognitive deficits emerging in several neurodegenerative pathologies (see Muchowski and Wacker 2005, for a review). For example, one main characteristic of patients suffering from Alzheimer's (AD), Parkinson's (PD), or Huntington's (HD) disease is the accumulation of protein aggregates in key brain regions for learning. The fact that each disease is cell-type, brain region- and misfolding protein-specific indicates, however, a common incapacity of molecular chaperones to prevent inappropriate aggregation of proteins or to mediate their final packaging, repair or degradation (Muchowski, 2002). This suggests, in turn, that over-expression of HSP might play a neuroprotective role which is independent from the nature of the disease. In agreement with this, ubiquitous neuroprotection has been reported following over-expression of Hsp70. For example, increased levels of Hsp70 protects neurons from Aβ42 mediated toxicity (Magrané et al., 2004) while an inverse relationship between aggregated tau and Hsp70 and Hsp90 levels has been detected both in the tau transgenic mouse modelling AD and in AD patients (Dou et al., 2003). Furthermore, in a *Drosophila*

model of PD, it has been shown that decreasing endogenous Hsc70 accelerates dopaminergic neuronal loss produced by α-synuclein aggregates while increasing the expression of human Hsp70 prevents it (Auluck et al., 2002). In a murine model of the same pathology, breeding α-synuclein transgenic mice with mice overexpressing Hsp70 significantly reduces the levels of high molecular weight α-synuclein prone to aggregate, and of detergent-insoluble alpha-synuclein species (Klucken et al., 2004). Moreover, Hsp70 induction by geldanamycin, a benzoquinone anisomycin that binds to Hsp90 and activates the heat shock response in mammalian cells, reduces both total α-synuclein and high molecular weight α-synuclein aggregates in vitro (McLean et al., 2004). Interestingly, geldanamycin also suppresses huntingtin protein aggregation in vitro in a dose-dependent manner (Sittler et al., 2001). Finally, recent epidemiologic studies in which HSP levels were assessed in different neuronal cell types taken from AD and PD patients revealed that neurons in the entorhinal cortex and hippocampus showed lower expression of Hsc70 and Hsp27 than dopaminergic neurons of substantia nigra. Because AD is fourfold more frequent than PD, it has been suggested that distinct levels of Hsc70 and Hsp27 confer to neuronal cell populations a variable buffering capacity against protein misfolding disorders that correlates with the relative frequencies of AD and PD in human populations (Chen and Brown, 2007).

CONCLUSION

During the last decade, significant contributions to the role of HSP70 in brain processes that regulate the modulation and consolidation of memory have emerged. A few years ago, it was believed that the function of these proteins was to allow cells to survive in lethal conditions. Advances in the analysis of the HSP70 cytoprotective function have progressively unveiled an unexpected role for these proteins in the mediation of intercellular signaling. In the field of learning, it is undoubted that the localization of HSP70 in the active zone of dendritic spines, where about 90% of excitatory synapses are formed, represents a strong argument in favor of their participation in the synaptic activity required for encoding and storing information. Nevertheless, further investigations are necessary to precise the details of their learning-specific involvement. For example, because of their presence in the PSD, one still open question is whether HSP70 merely optimize the holding/folding of proteins critical for neuronal plasticity or, alternatively, are intrinsic plasticity proteins. Also, there is evidence of a functional specialization of HPS70 according to their site of induction with intracellular proteins being involved in neuroprotection, and extracellular (membrane-associated) proteins exerting immunological functions (Johnson and Fleshner, 2006). Thus, delineating which class of HSP70 is mainly expressed following learning would serve to shed light on the primary sites (membrane or cytosol) of their learning-related activity. Finally, considering that rapid induction of HSP is controlled by heat shock transcriptional factors HSF, and that null mutant mice for the HSF-1 (Xiao et al., 1999) and HSF-2 (Wang et al., 2003)

genes have been generated, analyzing learning abilities in these null mutants would help to approach the function of HSP70 in learning more in depth. For example, since deleting the HSF-1 gene abolishes the heat shock response without affecting HSP basal expression, mice lacking the HSF-1 gene would aid in dissociating the respective weight of basal versus induced Hsp70 in memory. Differently, data showing that deleting the HSF-2 gene produces both an enlargement of the lateral and third ventricles and a reduction of hippocampus and striatum volume (Kallio et al., 2002), suggests a link between altered transcription of HSP and abnormal development of key brain areas for learning. Combining genetic, molecular and behavioral tools should rapidly increase our knowledge on how HSP70 interfere with acquisition processes and, hopefully, provide insights into the potential role of these proteins in preserving or improving cognitive processing in normal and pathological conditions.

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PART III

HEAT SHOCK PROTEINS AND EXERCISE PHYSIOLOGY

CHAPTER 14

HSP, EXERCISE, AND ANTIOXIDANTS

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Abstract: The heat shock proteins (HSP) are a family of highly conserved proteins with critical roles in maintaining cellular homeostasis and in protecting the cell from chronically and acutely stressful conditions. An increased expression of Hsp72 in skeletal muscle appears to be a part of the normal exercise response as well as training adaptation. Regular exercise offers protection against a number of chronic diseases, including type 2 diabetes and the basal expression of Hsp72 mRNA is suppressed in skeletal muscle of patients with type 2 diabetes. Moreover, mechanistic studies show that muscular Hsp72 protects against obesity-induced peripheral insulin resistance. Physical activity provokes an increased production of reactive oxygen species (ROS) and ROS is an inducer of Hsp72. The mechanisms whereby exercise and ROS regulate muscular Hsp72 expression are discussed. Anti-oxidant treatment with the vitamin E isoform γ-tochoferol is a potent inhibitor of exercise-induced expression of muscular Hsp72. This fact may contribute to explain the findings from large clinical studies that antioxidant supplementation may have detrimental effects on morbidity and mortality

Keywords: Physical activity; muscle; cytokines; physiology; vitamin; oxidative stress

Abbreviations: HSP, heat shock proteins ; Hsp72, seventy two kilo-Dalton Hsp ; IL-6, interleukin-6 ; L-NAME, nitro-L-arginine methyl ester ; rhIL-6, recombinant human IL-6 ; RNS, reactive nitrogen species ; ROS, reactive oxygen species ;

INTRODUCTION

Regular exercise offers protection against all cause mortality, primarily by protecting against atherosclerosis and type 2 diabetes (Blair et al., 2001). In addition, physical training is effective in the treatment of patients with ischaemic heart disease and

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type 2 diabetes (Pedersen and Saltin, 2006). A pivotal factor in the link between physical activity and health is exercise-induced regulation of gene expression within skeletal muscle itself and/or within other organs influenced by skeletal muscle signalling. Almost quiescent in resting myocytes, the mRNA levels of several cytokines increase quickly and robustly with exercise (Febbraio and Pedersen, 2002; Nieman et al., 2005; Steensberg et al., 2007). The cytokines undoubtedly have local paracrine effects, but are also secreted from the contracting myocytes and appear in the circulating blood and may change metabolism in other organs (Pedersen and Febbraio, 2008). Skeletal muscle contractions also initiate the regulation of genes with intrinsic effects in the myocytes. These include the heat shock protein 72 (Hsp72).

EXERCISE AND HEAT SHOCK PROTEINS

The heat shock proteins (HSP) are a family of highly conserved proteins with critical roles in maintaining cellular homeostasis and in protecting the cell from chronically and acutely stressful conditions (Lancaster and Febbraio, 2005). Subacute activation of HSP results in stress tolerance and cytoprotection against otherwise lethal exposures to stress-induced molecular damage (Morimoto, 1993; Chung et al., 2008). The induction of the HSP, therefore, may have broad therapeutic benefits in the treatment of various types of tissue trauma and disease (Chung et sal., 2008). In skeletal muscle, the most widely studied group of HSP is the 70-kDa family, which contains the constitutive Hsp73 and inducible Hsp72 forms (Madden et al., 2008; Febbraio and Koukoulas, 2000). Skeletal muscle Hsp72 expression increases in response to a bout of concentric exercise associated with no or minimal muscle damage (Febbraio and Koukoulas, 2000; Febbraio et al., 2002b; Khassaf et al., 2003). When HSP are located intracellularly, they work as chaperone-like proteins involved in folding of newly synthesized proteins, binding to denatured proteins, and translocation of proteins between subcellular compartments (Feder and Hofmann, 1999). Cell surface-bound HSP have been shown to facilitate migration and cytotoxicity by natural killer cells (Gastpar et al., 2005), whereas circulating HSP have pro-inflammatory actions and promote the release of proinflammatory cytokines from monocytes (Asea et al., 2000). Thus, intra- and extracellular Hsp72 have different functions. While intracellular Hsp72 confers cellular protection from subsequent stressors, extracellular Hsp72 appears to have a whole-body systemic role in antigen presentation and immunity.

An acute exercise bout stimulates an increase in both intra- and extracellular Hsp72. In response to acute exercise in vivo, the inducible 72-kDa member (Hsp72) has been shown to increase in the contracting muscle per se (Locke et al., 1990; Puntschart et al., 1996; Febbraio and Koukoulas, 2000), in several other tissues (Kregel, 2002), and in the circulation (Walsh et al., 2001). However, in a human exercise study, it was shown that the contracting muscle did not contribute to the increase in circulating Hsp72 since there was no difference in the femoral venous-arterial Hsp72 concentration at any time (Liu et al., 1999). We conducted an invasive

human exercise study in which we obtained blood samples from a brachial artery, a femoral vein, and the hepatic vein, and demonstrated that the increase in arterial Hsp72 was accounted for, at least in part, by a release from the hepatosplanchnic viscera, whereas contracting skeletal muscle did not release Hsp72 (Febbraio et al., 2002a). Long-term training and improved fitness increase the rate of availability of intracellular, but not extracellular, Hsp72 in response to stress. Thus, an increase of skeletal muscle Hsp72 content appears to be part of the normal exercise response as well as training adaptation (Yamada et al., 2008). Exercise-induced expression of intracellular Hsp72 (Febbraio and Koukoulas, 2000) is thought to be important for preservation of the intracellular environment during the oxidative and thermal stress of myocyte contraction (Smolka et al., 2000). In vitro and in vivo animal models have shown that an increased expression of intracellular Hsp72 is associated with improved cellular survivability and tolerance to stressors (Morimoto, 1993) and more specifically that Hsp72 protects against contraction-induced ROS formation (Smolka et al., 2000). The basal expression of Hsp72 mRNA is suppressed in skeletal muscle of patients with type 2 diabetes (Kurucz et al., 2002; Patti et al., 2001; Bruce et al., 2003) and it has been suggested that Hsp72 plays a role in obesity-induced peripheral insulin resistance (Chung et al., 2008). The role of muscular Hsp72 as an important mediator of the beneficial health effects of exercise makes it of interest to unravel mechanisms underlying the regulation of Hsp72 expression.

EXERCISE, HSP AND FREE OXYGEN RADICALS

Physical activity provokes an increased production of free radicals (Fig. 14.1) with the contracting skeletal muscle being a major source of both reactive oxygen species (ROS) (Davies et al., 1982; Jackson et al., 1985) and reactive nitrogen species (RNS) (Balon and Nadler, 1994). In vitro, HSP can be induced by ROS (Marini et al., 1996; Wallen et al., 1997) and by the mutagenic RNS compound peroxynitrite ($ONOO^-$) (Adrie et al., 2000), which is formed when superoxide (O_2^-) reacts with nitric oxide (NO). The tissue hosts a number of enzymes, e.g., superoxide dismutase and catalase and small molecule antioxidants, e.g., vitamins C and E, which offer some protection against oxidative stress-induceddamage to lipids (Alessio et al., 1988), proteins (Smolka et al., 2000) and DNA (Poulsen et al., 1996). It is increasingly recognized that oxidative stress is involved in the induction of HSP in the tissue, whereas exogenous supplementation with antioxidants may interfere with this adaptation. For example, α-tocopherol (a common isoform of vitamin E) has been shown to attenuate the increase of Hsp72 in leukocytes after treadmill running (Niess et al., 2002) whereas Khassaf et al. (2003) showed that supplementation with ascorbic acid (vitamin C) attenuates the exercise-induced increase of skeletal muscle Hsp72 while at the same time increasing baseline levels of Hsp72, superoxide dismutase, and catalase.

Seeing that the combination of ascorbic acid and α-tocopherol comprises a powerful antioxidant, both from an electrochemical (Buettner, 1993) and from an

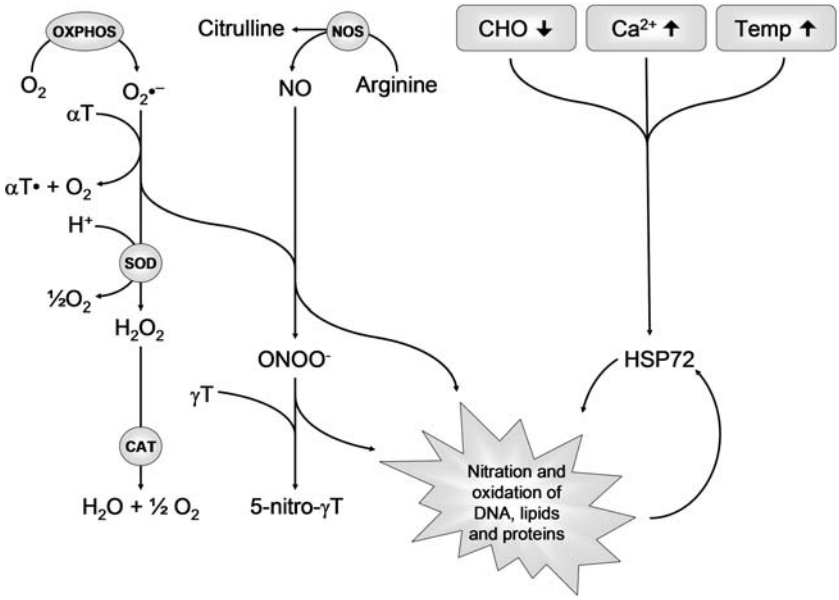

Figure 1. Schematic view of the possible mechanisms involved in the increase of Hsp72 in contracting skeletal muscle: increased oxidative phosphorylation (OXPHOS) leads to increased formation of the reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$). Superoxide can be removed by superoxide dismutase (SOD) and catalase (CAT) in a two-step reaction leading to the formation of O_2 and H_2O. In addition, small molecule antioxidants, e.g., α-tocopherol (α-T) and vitamin C, may neutralize superoxide by trapping the unpaired electrons. However, exercise is also associated with increased formation of reactive nitrogen species (RNS) from nitric oxide (NO) produced by nitric oxide synthase (NOS). Superoxide may cause oxidative stress directly or react with NO leading to the formation of peroxynitrite ($ONOO^-$), which is a mutagenic compound highly capable of nitration and oxidation of DNA, lipids and proteins. Of note, the vitamin E isoform γ-tocopherol (γT), but not α-tocopherol, is capable of neutralizing peroxynitrite. In order to protect cellular integrity, stress-inducible proteins such as the 72-kDa heat shock protein (Hsp72) act like chaperones, e.g. via binding to denaturated proteins. In addition to ROS and RNS, elevated temperature (Temp), decreased carbohydrate (CHO) availability, increased intracellular calcium levels (Ca^{2+}) as well as other exercise-related stressors of the cellular homeostasis have been shown to induce Hsp72.

experimental (Huang et al., 2002; Kanter et al., 1993) point of view, we hypothesized that the combined effect of the two would cause a marked attenuation of the exercise-induced Hsp72 mRNA and protein expression in skeletal muscle as well as circulating Hsp72. However, because α-tocopherol supplementation suppresses plasma levels of γ-tocopherol (Fig. 14.2), we also examined whether a combination of ascorbic acid, α-tocopherol, and γ-tocopherol would be a more potent inhibitor of the exercise-induced elevation of Hsp72 compared with ascorbic acid and α-tocopherol alone. Of note, γ-tocopherol, in contrast to α-tocopherol, is a potent scavenger in vitro of RNS, including peroxynitrite (Christen et al., 1997; Cooney

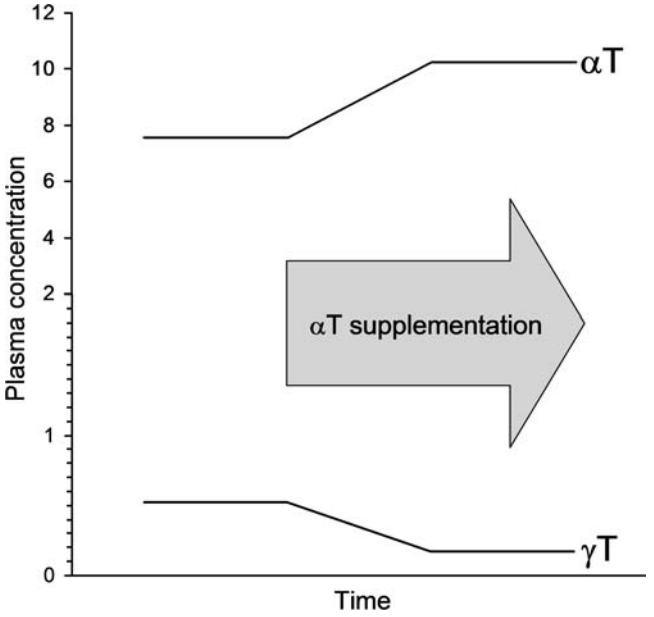

Figure 2. Schematic view of the effect of supplementation with α-tocopherol on plasma concentration of different vitamin E isoforms: in plasma, α-tocopherol (αT) is the predominant isoform of vitamin E. In response to daily supplementation with 400 IU of α-tocopherol – a dose employed in many human vitamin E supplementation studies – the plasma concentration of α-tocopherol (αT) increases further. In contrast, the plasma concentration of other tocopherols, e.g., γ-tocopherol (γT) is suppressed markedly. Since γ-tocopherol appears to have scavenging properties different from those of α-tocopherol, supplementation with α-tocopherol alone may cause increased formation of other highly reactive species such as peroxynitrite. Concentrations are shown as μg/ml.

et al., 1993), and an inhibitor of cyclooxygenase activity (Jiang et al., 2000), thus possessing potential anti-inflammatory qualities.

We hypothesized that supplementation with antioxidant vitamins C (ascorbic acid) and E (tocopherol) would attenuate the exercise-induced increase of Hsp72 in the skeletal muscle and in the circulation (Fischer et al., 2006). In a randomised study, we allocated young healthy men into three groups receiving one of the following oral supplementations: RRR-α-tocopherol 400 IU/day + ascorbic acid 500 mg/day, RRR-α-tocopherol 290 IU/day + RRR-γ-tocopherol 130 IU/day + ascorbic acid 500 mg/day, or placebo. After 28 days of supplementation, the subjects performed 3 h of knee extensor exercise at 50% of their maximal power output. The main finding of this study was that oral supplementation with vitamins C and E inhibited the exercise-induced increase of Hsp72 in contracting skeletal muscle and in the circulation, but only when the supplementation included the vitamin E isoform γ-tocopherol. Possible explanations for the differential effect of the two combinations of antioxidants were considered: either the depletion of γ-tocopherol when

providing supplementation with α-tocopherol partly counteracts the overall antioxidant effect indicated by decreased lipid peroxidation or, alternatively, the effect of surplus γ-tocopherol may specifically inhibit Hsp72 in response to exercise. Although not conclusive, the association between high plasma γ-tocopherol levels and low serum Hsp72 levels supported the latter point of view. In vitro, a combination of α-, δ-, and γ-tocopherol is a more efficient inhibitor of lipid peroxidation in erythrocytes compared with α-tocopherol alone (Liu et al., 2002). The combination containing γ-tocopherol, however, did not appear to be a more efficient antioxidant than the combination without γ-tocopherol, at least when using the F_2-isoprostane 8-iso-$PGF_{2\alpha}$ as marker of lipid peroxidation. Therefore, the marked effect of γ-tocopherol may be due to the fact that the latter, but not α-tocopherol, is a scavenger of peroxynitrite (Christen et al., 1997; Wolf, 1997), which at least in vitro is a highly potent stimulus of HSP synthesis in monocytes (Adrie et al., 2000). A human in vivo study did not find support for a direct effect of NO in mediating the exercise-induced increases in muscular Hsp72: Healthy males participated in an exercise study. They were randomised to (1) a control group who performed exercise; (2) a group who performed exercise while they received infusion with an NO synthase inhibitor (nitro-L-arginine methyl ester, L-NAME, 5 $mg \cdot kg^{-1}$); and (3) a group, who received femoral artery infusion of the NO donor nitroglycerin (NTG, 1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$). While the study showed that NO production contributes to the regulation of gene expression in muscle during exercise of some genes (interleukin-6 (IL-6), interleukin-8 (IL-8), heme oxygenase-1 (HO-1)), this was not the case for Hsp72 (Steensberg et al., 2007). Therefore, although ROS appears to be one important stimulus for an increased HSP synthesis, it is difficult to draw firm conclusions with regard to RNS (Wallen et al., 1997).

OTHER MEDIATORS OF EXERCISE-INDUCED REGULATION OF HSP

Several other exercise-related stressors of the cellular homeostasis have been shown to induce HSP (Fig. 14.1) either in vivo or in vitro: elevated temperature (Mizzen and Welch, 1988), decreased glucose availability (Bergstedt et al., 1993), increased intracellular calcium levels (Welch et al., 1983), increased adrenergic stimulation (Paroo and Noble, 1999), ischemia (Marber et al., 1995), and hypoxia (Hammerer-Lercher et al., 2001). In the beginning of this millennium, we demonstrated that contracting human skeletal muscle produces and releases IL-6 into the circulation (Pedersen and Febbraio, 2008). To determine whether the IL-6 was an inducer of Hsp72 gene expression in skeletal muscle, we infused recombinant human IL-6 (rhIL-6) and placebo into healthy volunteers. Hsp72 gene expression did not increase above resting levels in controls, but increased during and following infusion of rhIL-6 for 3 h. These data demonstrate that IL-6 rapidly induces Hsp72 gene expression in human skeletal muscle (Febbraio et al., 2002c). In another study, we were able to show that the exercise-induced release of IL-6 from an exercising limb was inhibited when the subjects were treated with anti-oxidants (vitamin C + α-tocopherol) (Fischer et al.,

2004). While the latter study clearly demonstrated that anti-oxidant treatment has a marked effect on the release of IL-6 from working muscle, unpublished data from our group (Fischer et al.) show that although the effect of anti-oxidants on HSP expression (Fischer et al., 2006) could be ascribed to a specific effect of the vitamin E isoform, γ-tocopherol, the effect on IL-6 release was found using both RRR- α-tocopherol alone and in combination with RRR- γ-tocopherol, making it less likely that the anti-oxidant effect on Hsp72 was mediated via an effect of muscle-derived IL-6.

CONCLUSION

Increasing evidence suggests that intracellular expression of HSP has numerous protective effects for health. In this context, increased muscular expression of HSP may represent one among several links between physical exercise and health. ROS appears to play a major role in mediating muscle adaptation to exercise, including increased muscular Hsp72 expression (McArdle et al., 2004; Jackson, 2005; Fischer et al., 2004). Apparently antioxidants attenuate some of the normal physiological responses to non-damaging exercise. Therefore, the use of antioxidant supplementation, and not the least vitamin E, may be less desirable from a long-term health perspective. This point of view is partly supported by large studies showing no or even a detrimental effect of antioxidant supplementation on morbidity and mortality (Vivekananthan et al., 2003; Heart Protection Study Collaborative Group, 2002; Skibsted et al., 2006).

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CHAPTER 15

EXERCISE INTENSITY AND DURATION AFFECT BLOOD-SOLUBLE HSP72

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Abstract: Extracellular Hsp72 (eHsp72) is elevated during and after acute bouts of exercise. Concentrations of eHsp72 in plasma or serum are dependent on the duration and intensity of exercise. Active secretory processes rather than passive release due to cell damage are considered to function in the exercise-induced release of eHsp72. Up-regulation of eHsp72 in the circulation following acute exercise may contribute to improved immune function; eHsp72 increases stress resistance after binding to stress sensitive recipients, signals tissue destruction and danger to inflammatory cells, and aids in immunosurveillance by transporting intracellular peptides to distant immune cells. It is uncertain whether this exercise-mediated mechanism for eHsp72-regulated activation of the immune system helps to prevent immunologic diseases

Keywords: Exercise; acute response; immune system; blood; extracellular heat shock protein 72

Abbreviations: APC, antigen-presenting cells; ATP, adenosine triphosphate; CCRS, chemokine receptors; eHsp72, extracellular Hsp72; HSP, heat shock proteins; Hsp72, seventy two kilo-dalton HSP; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; TLR, toll-like receptor

INTRODUCTION

The exercise-mediated regulation of extracellular Hsp72 (eHsp72) in the plasma or serum of peripheral blood is a very interesting aspect of exercise immunology.

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eHsp72 has been found during and after acute bouts of exercise (Walsh et al., 2001; Febbraio et al., 2002, 2004; Lancaster et al., 2004; Peake et al., 2005; Fehrenbach et al., 2005) and its levels are elevated under pathological conditions (Pockley, 2001). It is clear that eHSP can play a role as pro-inflammatory immune effectors (Millar et al., 2003; Daniels et al., 2004), but it is unclear whether eHsp72 plays a role as a pro-inflammatory mediator or in chaperoning proteins to prevent aggregation or proteolysis of damaged proteins due to exercise. This chapter will review exercise-induced blood-soluble Hsp72, and discuss current hypotheses on the mechanisms underlying Hsp72 secretion, as well as their implications in blood-soluble Hsp72 regulation.

EXERCISE INTENSITY AND DURATION FOR SECRETION OF HSP72

Walsh et al. first reported an exercise-induced increase in eHsp72 (Walsh et al., 2001). Subsequently, other exercise-related studies demonstrated that the concentrations of eHsp72 in plasma are dependent on the duration and intensity of exercise (Fehrenbach et al., 2005; Peake et al., 2005) (Figure 1 and Table 15.1), that eHsp72 elevation is accompanied by parallel increases in cytokines (Fehrenbach et al., 2002; Peake et al., 2005) and biomarkers for oxidative stress (Banfi et al., 2005), and that vitamin E and a specific isoform in particular blunts this exercise-induced increase

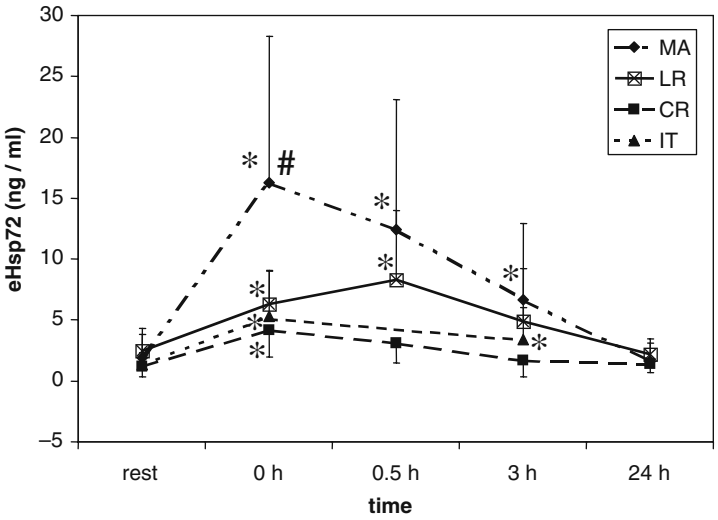

Figure 1. Changes in extracellular HSP72 (at rest, 0h, 0.5h, 3h, and 24 h after exercise) in the plasma of endurance athletes were dependent on intensity and duration of exercise. Marathon running (MA), long running (LR), continuous running (CR) and interval training (IT) were compared. Data are presented as means ± SD. ∗denotes significant differences between pre- and post-exercise data, $p<0.05$; originally published in Fehrenbach (2005b).

Table 1. The intensity and duration of exercise affect extracellular HSP72. Comparison of the fold increases in HSP72 in blood in previous investigations

Maximal x-fold increase	Intensity (%VO_2 max)	Duration (min)	Type	Localization	Comment	References
Exhaustive exercise						
14	65	260	Marathon run	Plasma		Fehrenbach et al. (2005b)
6	60	120	Long treadmill run	Plasma		
4	75	60	Continuous treadmill run	Plasma		
5	83	35	Extensive interval training program	Plasma		
16		539	100-km run	Plasma	Different subjects	Gomez-Merino et al. (2006)
6		504	Long-distance triathlon	Plasma		
12	80	60	Treadmill run	Plasma	Same subjects	Fehrenbach et al. (2005b)
2	60	60		Plasma	Same subjects	
2.7	70	90	Treadmill run	Plasma		Horn et al. (2007)
Different localization						
2	62	120	Bicycle	Leg		Febbraio et al. (2002)
8	~65	120	Bicycle	Liver, arterial		Febbraio et al. (2004)
4				Liver, hepatosplanchnic		
2	60	180	Bicycle	Brain, arterial		Lancaster et al. (2004)
2.5				Brain, venous	Individually different	
Effect of ambient temperature and humidity						
1.6	42.5	120	Bicycle	Serum	Day 1	Marshall et al. (2006)

Table 1. (continued)

Maximal x-fold increase	Intensity (%VO_2 max)	Duration (min)	Type	Localization	Comment	References
1.2			Hot (38C), humid (60%)		Day 2	
1.1	56	100	Walk	Serum	Day 1	Yamada et al. (2007)
1					Day 10 (heat acclimatization)	
1	90	90	Bicycle	PBMC lysate	Active	Lovell et al. (2007)
1.8			Hot bath 40C		Passive	
Different types of exercise						
1.5	60	60	Treadmill running	Plasma		Peake et al. (2005)
1.9	85	60	Treadmill running			
2.5	60	45	Downhill running			
1.4	40% of maximal isometric strength		1st Bout of elbow flexor	Plasma	Same subjects	Hirose et al. (2004)
1.2	6 set of 5 action 2 min interval		2nd Bout of the same exercise	Plasma		
Effect of supplementation						
2.5	70	90	Bicycle	Plasma	Control	Whitham et al. (2006)
2					Caffeine supplementation	
3∼4	50% of the maximal power	180	Knee extensor	Serum	Vitamin CEα	Fischer et al. (2006)
1					Vitamin CE$\alpha\gamma$	
3∼4					Control after 28 days of supplementation	

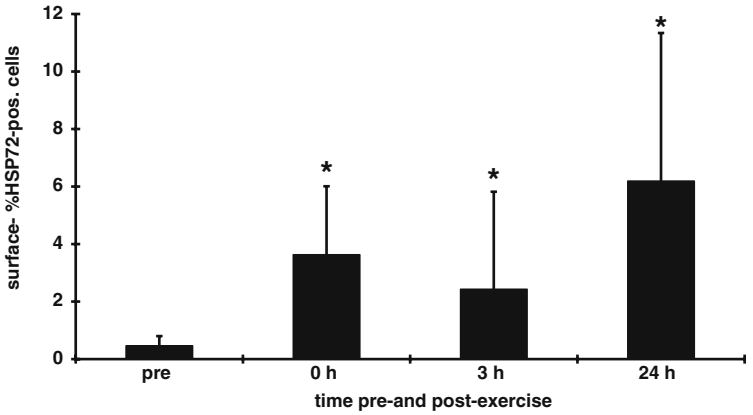

Figure 2. Expression of HSP72 in leukocytes increased after strenuous endurance exercise (pre-, 0h, 3h, 24 h after marathon running). A further increase in the percentage of HSP72-positive leukocytes at 24 h after exercise was observed. Data are presented as percent positive cells (%); originally published in Fehrenbach et al. (2000a).

of eHsp72 (Niess et al., 2002; Fischer et al., 2006). In addition, glucose ingestion attenuates the exercise-induced increase in eHsp72 (Febbraio et al., 2004). eHsp72 increases adaptation to exercise in hot, humid environments (Marshall et al., 2006). However, the effect of heat acclimation on Hsp72 is controversial (Marshall et al., 2006; Yamada et al., 2007). Hsp72 expression on the surface of tissues and cells also changes due to exercise. The changes in Hsp72 expression by half marathon running are shown in Figure 2. This strenuous endurance exercise stimulated Hsp72 expression in leukocytes, and increases in the percentage of Hsp72-positive leukocytes were observed at 24 h after exercise. Hsp72 positive monocytes and granulocytes in trained athletes were significantly lower when compared with the corresponding cells in untrained individuals (Fehrenbach et al., 2000a).

POTENTIAL MECHANISMS OF EXERCISE-INDUCED EHSP72 ELEVATION

Passive or Active Mechanisms?

The mechanisms for excretion from possible intracellular storages sites remain controversial. Recent work from several groups has suggested that HSP is released by both, passive (necrotic) and active mechanisms (Basu et al., 2000; Schild and Rammensee, 2000; Febbraio et al., 2002; Asea, 2005). During exercise, the release of Hsp72 from damaged cells only partially contributes to circulating eHsp72 levels. A comparative study between endurance exercise of varying intensity and duration

revealed that the run with the highest eHsp72 levels in plasma was associated with the most pronounced concentrations of creatine kinase, which is a prominent marker of tissue damage (Fehrenbach et al., 2005). On the other hand, release from injured tissue can largely be excluded, as eHsp72 increases after exercise even in the absence of enhanced plasma creatine kinase levels (Lancaster et al., 2005b). Moreover, despite the absence of liver cell damage, hepatosplanchnic release of Hsp72 was confirmed after exercise (Febbraio et al., 2002). At present, active secretory processes rather than passive release due to cell damage, are considered to function in the exercise-induced release of eHsp72 (Lancaster and Febbraio, 2005a, b).

How Is Hsp72 Released?

While the molecular mechanisms of HSP synthesis are well documented, it is unclear how HSP are released into the extracellular milieu. HSP lack the leader sequence normally involved in secretion, and their release involves non-classical secretion mechanisms. A number of different models for releasing extracellular Hsp72 without necrosis have been proposed. These involve (i) lysis of the originating cell (Jonson et al., 2005), (ii) release through vesicles by a blebbing mechanism, followed by subsequent lysis of vesicles (Lancaster and Febbraio, 2005a), and (iii) secretion through endolysosomes and release when the endolysosome fuses with the cell surface (Mambula and Calderwood, 2006) (Figure 3).

Various hormones increase during exercise. The results of these previous studies have indicated that a close relationship exists between neuroendocrine activation during stress and secretion of Hsp72. It has been suggested that norepinephrine in rats (Jonson et al., 2005) and epinephrine in humans (Whitham et al., 2006) have a prominent role in the stimulation of eHsp72. However, Hsp72 content in the cell does not always reflect the magnitude of extracellular Hsp72 (Lancaster and Febbraio, 2005b). Stress-induced increases in Hsp72 are seen very rapidly in plasma, for example, tail shock stress increases Hsp72 in blood within 10 min of stress onset (Fleshner et al., 2003), and treadmill running under 65% VO_2 max increased Hsp72 in blood within 30 min of stress onset (Febbraio et al., 2002). It is thus believed that intracellular Hsp72 is the potential source of extracellular Hsp72, as the release may not depend on the translation of new intracellular protein (Fleshner et al., 2003).

In the non-classical protein transport pathway, lipid rafts are specialized membrane microdomains that are formed within the exoplasmic leaflet of the Golgi membrane, and may play a role in Hsp72 exocytosis (Broquet et al., 2003); however, the effect is controversial due to cytotoxicity (Lancaster and Febbraio, 2005b). Exosomes, which are small membrane vesicles secreted by various cell types including B cells (Clayton et al., 2005), T cells (Blanchard et al., 2002), dendritic cells (Segura et al., 2005), mast cells (Skokos et al., 2003), epithelial cells (van Niel et al., 2001) and peripheral blood mononuclear cells (PBMC) (Lancaster and Febbraio, 2005a), may provide a secretory pathway allowing cells to actively release specific

HSP. Lancaster et al. demonstrated that exosomes gradually increase in both the culture medium (RPMI 1640, 0% fetal bovine serum) and PBMC cell culture under basal incubation (37°C) in a time-dependent manner, while the Hsp72 content of exosomes also tended to increase (Lancaster and Febbraio, 2005a).

A potential alternative mechanism for the secretion of Hsp72 in non-classical pathways is the endolysosome pathway (Mambula and Calderwood, 2006; Mambula

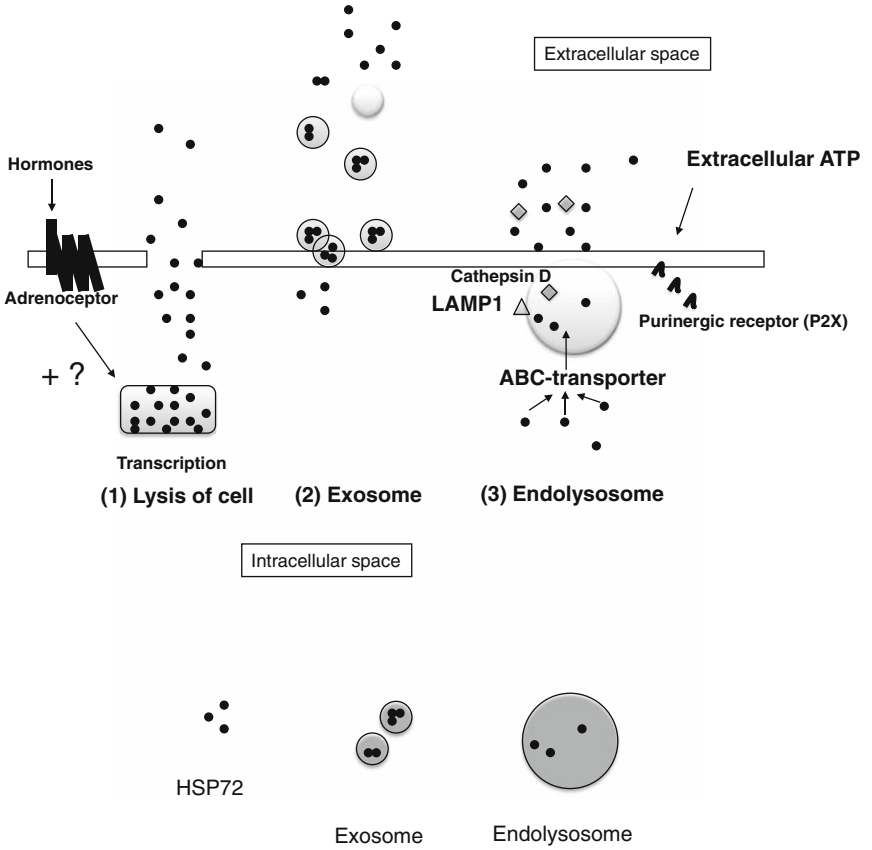

Figure 3. Non-classical pathways of HSP72 release. (1) Lysis of cell of origin: HSP72 spills over into blood in a similar manner as plasma norepinephrine. Thus, intracellular levels of HSP72 reflect extracellular HSP72. (2) Release from vesicles by a blebbing mechanism followed by subsequent lysis of vesicles, such as exosomes and lipid rafts. (3) Secretion through endolysosomes and release when the endolysosomes fuse with the cell surface: This type of HSP72 secretion involves entry of HSP72 into endolysosomes through ABC-family transporters, where they co-localize with intravesicular cathepsin D. Extracellular ATP coordinates this lysosomal pathway through purinergic receptors, and these organelles are then transported to the cell surface. Subsequent fusion of HSP72-containing endolysosomes with the cell surface results in the localization of LAMP1 on the plasma membrane and the release of HSP72 along with other proteins, such as cathepsin D.

et al., 2007) (Figure 3). Hsp72 release involves transit through the endolysosomal compartment - the entry of Hsp72 into this secretory compartment appears to involve ABC-family transporter proteins – and this is followed by triggering stress-induced Hsp72 release through extracellular adenosine triphosphate (ATP). Downhill running induces increases in eHsp72 (Peake et al., 2005), whereas eHsp72 dose not increase after eccentric exercise such as elbow flexors (Hirose et al., 2004), despite both types of exercise inducing muscle damages. This may be partially explained by the endolysosome mechanism: extracellular ATP regulates Hsp72 release from ABC-family transporters, and thus muscle damage does not contribute to increased eHsp72; instead, eHsp72 increases with extracellular ATP.

Potential Sources of eHsp72

There are several potential sources for the release of Hsp72 into circulation in response to exercise. These include skeletal muscle, brain, heart, leukocytes and the hepatosplanchnic tissues (Moseley, 2000; Walsh et al., 2001; Febbraio et al., 2002; Hunter-Lavin et al., 2004; Lancaster et al., 2004; Fehrenbach, 2005). In all of these tissues or cells, the concentration of HSP is up-regulated by exercise. Skeletal muscle is the most likely tissue source for eHsp72 in exercise. However, the increase in eHsp72 in serum precedes the increase of Hsp72 mRNA and protein in muscle (Walsh et al., 2001). Furthermore, eHsp72 can be found in arterial, but not venous, blood flow in the contracting leg model (Febbraio et al., 2002). Thus, human skeletal muscle does not appear to secrete Hsp72. On the other hand, in another study, it was revealed that release from human hepatosplanchnic tissue substantially contributes to eHsp72 concentrations in circulation (Febbraio et al., 2002). Significant exercise-induced increases in Hsp72 in the liver have also been detected in animal models (Salo et al., 1991; Kregel and Moseley, 1996). Moreover, release of Hsp72 from the human brain is induced by exercise (Lancaster et al., 2004). Leukocytes may be another source, as they are able to actively secrete Hsp72 (Hunter-Lavin et al., 2004) and their intracellular Hsp72 expression is increased by exercise (Fehrenbach et al., 2000a, b) (Figure 2). P2X receptor is ubiquitously expressed and belongs to a family of ligand-gated channels that are activated by extracellular ATP, which induces multimerization of P2X subunits into trimers and hexamers, thereby allowing transmembrane passage of cations and small molecules (Di Girolamo et al., 2005). P2X7 receptors are expressed in human including glia cells (Pannicle et al., 2000), macrophages (Jiang et al., 2000) and lymphocyte (Gu et al., 2000). Human P2X6 receptor, not P2X7, is heavily expressed in skeletal muscle (North, 2002). The receptor in liver is unusual for its high sensitivity to ATP, and the properties do not coincide with any of those yet studied by heterologous expression (North, 2002). Thus, P2X receptors in the endolysosomal pathway may be related to the source for Hsp72 release into circulation in response to exercise.

FUNCTIONAL CONSEQUENCES IN IMMUNE SYSTEM

Functionally, extracellularly localized and surface-bound HSP plays a major role in the activation of the immune system and may also be of importance for the immune response to exercise (Asea, 2003; Radons and Multhoff, 2005) (Figure 3). HSP in general, and Hsp72 in particular, are highly potent danger signals according to the danger theory of Matzinger (2002). They activate the immune system depending on their localization and protect cells against potentially lethal stresses (Campisi et al., 2003; Fleshner et al., 2003; Fleshner and Johnson, 2005). There are several possibilities for how eHsp72 is involved in the regulation of the immune response. (i) Internalization of Hsp72-peptide complexes by antigen-presenting cells (APCs) stimulates cross-presentation of HSP-chaperoned peptides in context with MHC class I molecules, thus activating the specific immune system via CD8-positive cytotoxic T lymphocytes and mediating a tumor-specific immune reaction (Doody et al., 2004). (ii) Unusual localization of eHsp72 on the surface of stressed cells provides a target structure to directly activate the migratory and cytolytic capacity of NK cells (Gastpar et al., 2004). HSP expression on the surface of tumor cells is also a recognition structure for NK cells (Multhoff, 2002). (iii) Binding of eHsp72 alone on specific surface receptors may induce non-specific immune stimulation and activates intracellular signaling cascades that result in a pro-inflammatory cytokine response (Asea et al., 2000; Vabulas et al., 2001). Additional cell surface proteins have been identified as receptors for Hsp72, including the scavenger receptor (SR) family, such as the C-type lectin receptor LOX-1 (Delneste et al., 2002), CD94, and SR-A, the low-density lipoprotein receptor-related protein/β_2-microglobulin CD91 receptor, the Toll-like receptor (TLR) 2 and 4, CD40, and chemokine receptors (CCRS) such as CCR5 (Floto et al., 2006), which are associated with endocytosis, phagocytosis and/or are involved in the induction of the pro-inflammatory response. In a study on the changes in cells expressing eHsp72 on their cell surface through these receptors following exercise (Horn et al., 2007), no effect was seen on the proportion or absolute count of eHsp72-positive monocytes ($CD14^+$) or on the proportion of eHsp72-positive NK cells ($CD3^-/CD16^+/CD56^+$), although exercise increased plasma Hsp72 concentrations in an in vivo study (Horn et al., 2007). This may be because the conserved c-type lectin domain was not the sole determinant required for Hsp72 interaction (Theriault et al., 2005; Calderwood et al., 2007), although c-type lectins may play a significant role in the interaction between Hsp72-expressing tumor cells and NK cells (Multhoff, 2002).

CONCLUSION

Up-regulation of eHsp72 in the circulation following acute exercise has been observed, and its levels are dependent on the duration and intensity of exercise. eHsp72 may also contribute to improved immune function. It is uncertain whether

eHsp72-regulated activation of the immune system by exercise prevents immunologic diseases. At present, however, these mechanisms remain speculative. To date, there has been only one study reporting elevated plasma Hsp72 concentrations in elite soccer players vs. sedentary controls (Banfi et al., 2005). Systematic investigation of the influence of regular training on plasma concentrations of eHsp72 in differently trained subjects as well as active or inactive patients is a promising future task.

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CHAPTER 16

ULTRA MARATHON RACE COMPETITION AND IMMUNE FUNCTION

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Abstract: This chapter reviews key exercise immunology findings from five years of research on 350 athletes in the 160-km Western States Endurance Run (WSER), a point-to-point trail run in the Sierra Nevada Mountains of northern California. Until this series of studies on WSER ultra-marathoners, little was known regarding the response of the immune system to severe exertion. This chapter describes how the immune system responds to running 160 km and underlying mechanisms including oxidative stress, muscle damage, inflammation, endotoxemia, and ibuprofen use

Keywords: Cytokines; running; immune function; ibuprofen; oxidative stress; inflammation; neutrophils

Abbreviations: ALT, alanine aminotransferase; AST, asparate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; CK, creatine kinase ; CRP, C-reactive protein; DOMS, delayed onset of muscle soreness; G-CSF, granulocyte colony-stimulating factor; IgA, immunoglobulin A; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migration inhibitory factor; MIP-1β, macrophage inflammatory protein 1 beta; NK, natural killer; NSAIDS, Non-steroidal anti-inflammatory drugs; RPE, ratings of perceived exertion; TNF-α, tumor necrosis factor-alpha; URTI, upper respiratory tract infections; WSER, Western States Endurance Run

INTRODUCTION

Athletes participating in prolonged and intensive exercise such as marathon and ultra marathon race events experience acute physiological stress reflected by muscle microtrauma, oxidative stress, and systemic inflammation (Akerström and

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Pedersen, 2007; Nieman, 2007; Suzuki et al., 2002). Concomitant with these stressors are widespread perturbations in innate and adaptive immunity including decreases in natural killer (NK) cell cytotoxic activity, granulocyte respiratory burst activity, nasal and salivary IgA (sIgA) secretion, delayed type hypersensitivity, and mitogen-induced lymphocyte proliferation, as well as extensive alterations in circulating immune cell populations (Bruunsgaard et al., 1997; Nielsen and Lyberg, 2004; Nieman, 2000a; Peake, 2002). This period of decreased host protection is often followed by elevated rates of upper respiratory tract infections in the athletes 1–2 weeks after competition (Nieman, 2000b; Peters and Bateman, 1983). Efforts to link the immunological perturbations with increased infection risks have however, been relatively unsuccessful (Gleeson, 2006).

The immunology of prolonged and heavy exertion of 5 h duration and longer is relatively unstudied compared to exercise bouts of shorter duration including marathons (Nieman et al., 2002b, 2004). This chapter summarizes data collected from 350 ultramarathon runners during a five-year period at the 160-km Western States Endurance Run (WSER). Portions of these data have been published in a series of papers, but this chapter is a collective analysis and summary of the entire data set (Dumke et al., 2007; Henson et al., 2008; McAnulty et al., 2007a, b; Nieman et al., 2003b, 2005, 2006a, b, c, 2007; Quindry et al., 2008).

Table 1 summarizes subject characteristics for all male and female subjects in these studies. In general, the data indicate that these runners (age range, 19–69 years) were deeply committed to training for and competing in ultra-marathons. A stepwise, multiple regression model showed that the 160-km WSER race time (h) could be predicted by this equation: 12.57 + (0.126 × age) + (2.56 × gender) + (0.285 × BMI) – (0.01931 × km/week) ($R^2 = 0.23$) (male gender=1, female=2). Thus race time for a 20 year old male with a 22.0 BMI, and a training distance of 130 km/week would be estimated at 21.4 h compared to 28.5 h for a 55 year-old male with a 27 BMI and 65 km/week training distance.

Table 1. Subject characteristics for 350 competitors in the 160-km Western States Endurance Run[a]

Variable	Males ($N = 279$)	Females ($N = 71$)	*P*-Value
Age (years)	47.1±0.5	43.5±0.8	0.001
Stature (m)	1.78±0.004	1.63±0.007	<0.001
Body mass (kg)	73.0±0.5	55.3±0.7	<0.001
Body mass index ($kg \cdot m^2$)	22.9±0.1	20.7±0.2	<0.001
Body composition (%)	10.7±0.4	18.9±0.6	<0.001
Training distance (km/week)	81.2±1.6	85.2±3.1	0.169
Years of training	15.6±0.5	13.5±1.0	0.087
Ultramarathon races run	39.2±2.9	29.0±4.7	0.067
Race time (h)	25.9±0.2	27.4±0.4	0.002

[a]Competition was composed of males = 80%; females = 20%. Data is mean ± SE.

THE 160-KM WESTERN STATES ENDURANCE RUN

To enter one of the five WSER studies, subjects must have qualified for the 160-km WSER (held in late June each year) by completing a 160-km race in less than 24 h, or a 100-km race in 12–13 h, depending on age. The 160-km Western States Endurance Run is a point-to-point trail run in the Sierra Nevada Mountains of northern California, and is regarded as one of the most arduous organized running events in the United States (Nieman et al., 2003b). The race starts at Squaw Valley, California (1,890 m altitude), and finishes at Auburn, California (366 m). The trail race course ascends 777 m to Emigrant Pass (2,668 m, the highest point) within the first 7 km and then passes through remote and rugged territory to Auburn. The total altitude gain and loss during the race is 5,500 and 6,700 m, respectively. The race starts at 5:00 am, and runners have to reach the finish line within 30 h to be eligible for an award. Up to half of the trail is traveled by some runners at night. A staff of over 1,300 volunteers supports the runners and works 26 aid stations, including 11 medical check points.

MAJOR FINDINGS FROM THE WSER RESEARCH PROJECTS

This chapter will review four key findings from the WSER research projects:

1. Twenty-four percent of ultra-marathoners studied reported upper respiratory tract infections (URTI) during the 2-week period following the 160-km WSER. The best predictor of URTI was a low-post WSER salivary IgA secretion rate.
2. Plasma cytokine levels rose to high levels during the WSER, with the greatest fold increases occurring for IL-6 ($\sim$130$\times$), IL-10 ($\sim$30$\times$), G-CSF ($\sim$25$\times$), IL-8 ($\sim$9$\times$), and IL-1ra ($\sim$7$\times$).
3. Increases in plasma cytokine levels during the WSER were highly variable between runners, with the largest increases measured in those experiencing the greatest muscle damage (CPK) and inflammation (CRP). Surprisingly, oxidative stress during the WSER was modest and not related to elevations in plasma cytokines.
4. Ibuprofen use was common among WSER competitors (70%). However, data indicate that ibuprofen users compared to nonusers experienced the same degree of muscle damage and soreness, and was linked to more endotoxemia, inflammation, kidney dysfunction, and higher plasma cytokines.

UPPER RESPIRATORY TRACT INFECTIONS

The majority of WSER endurance athletes reported that they felt protected from URTI compared to their sedentary peers. As depicted in Figure 1, 81% of WSER athletes surveyed claimed lower URTI rates in response to this question: “Compared

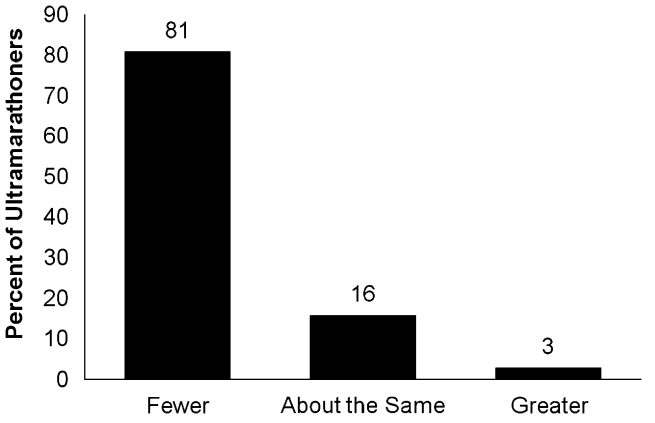


Figure 1. Survey responses by WSER athletes in response to this question: "Compared to others who do no run or exercise do you feel that you generally have _____ episodes of sickness with the common cold or flu?

to others who do no run or exercise do you feel that you generally have _____ episodes of sickness with the common cold or flu? However, this training-related protection from URTI was diminished during the 2-week period following the summer 160-km race, with 24% reporting URTI (Nieman et al., 2006a). This rate is higher than the 13% recorded during the week following the Los Angeles Marathon held during late winter (Nieman et al., 1990).

Prolonged and intensive exertion causes numerous changes in immunity that reflect physiologic stress and immunosuppression, and an increased risk of URTI (Nieman, 2000b, 2007; Nieman et al., 1990; Ostrowski et al., 2000; Palmer et al., 2003). There are multiple ways to measure immune dysfunction in endurance athletes following competitive marathon and ultramarathon race events. At the WSER, samples collected from the athletes must be processed and stabilized for analysis later on at distant laboratories. Outcome measures that work within this context include blood leukocyte and lymphocyte subset cell counts, granulocyte oxidative burst activity, salivary IgA output, plasma cytokine levels, and blood leukocyte cytokine mRNA expression. This chapter will summarize some of the important discoveries of immune dysfunction in WSER athletes. For example, in a subset of the WSER athletes, granulocyte oxidative burst activity was decreased by ~50% following the 160-km race (Henson et al., 2008) (Figure 2).

Salivary IgA secretion rate has emerged as a potential marker of increased infection risk in endurance athletes (Gleeson et al., 1999, 2000; Gleeson and Pyne, 2000). The secretory immune system of the mucosal tissues of the upper respiratory tract is considered the first barrier to colonization by pathogens, with IgA the major effecter of host defense. Individuals with selective IgA deficiency experience a high incidence of URTI, and a significant relationship between sIgA concentration

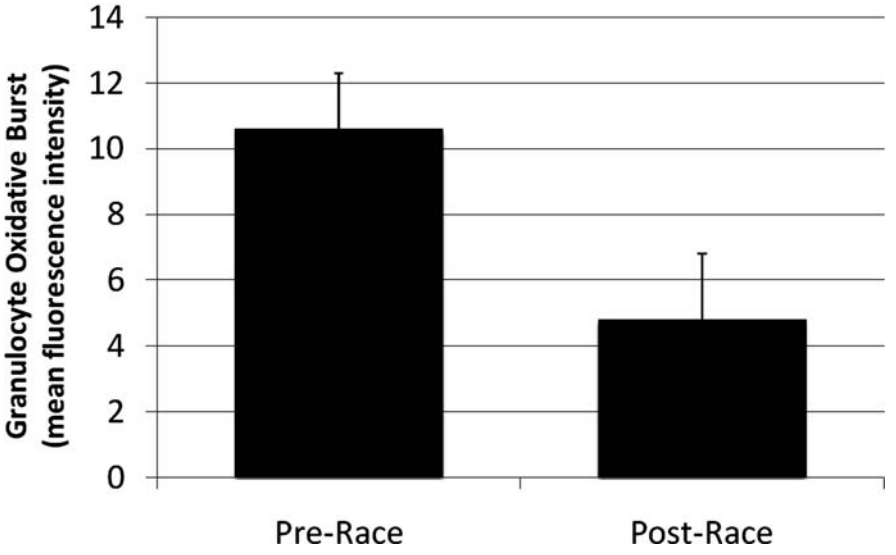


Figure 2. Pre- and post-WSER levels of granulocyte oxidative burst activity

and URTI incidence has been reported (Lehtonen et al., 1987). Thus monitoring of mucosal immune parameters during critical periods of training and competition has been promoted as useful in predicting URTI risk in athletes (Gleeson and Pyne, 2000).

Following intensive and prolonged exercise of more than 90 min duration by endurance athletes, the total sIgA output decreases in athletes. This was first reported by Tomasi et al. (1982) in eight elite Nordic skiers following a national cross-country ski race competition, and later confirmed in studies of cyclers, marathon runners, triathletes, and other athletes (Krzywkowski et al., 2001; Nieman et al., 2002a; Palmer et al., 2003). Steerenberg et al. (1997) reported that salivary flow rate and total sIgA output (but not sIgA concentration) were reduced in 42 triathletes following an Olympic-distance triathlon race event. Little or no change in sIgA output has been measured in athletes following low-to-moderate intensity exercise of an intermittent nature (e.g., tennis drills or soccer play), indicating that the combination of high intensity and prolonged duration is necessary before significant decreases in this immune parameter are measured.

Salivary IgA secretion rate decreased by nearly half in the WSER ultramarathon runners following the 160-km race (Henson et al., 2008; Nieman et al., 2006b). Nearly one in four runners reported an URTI episode during the 2-week period following the race, and the decrease in sIgA secretion rate was significantly greater in these runners (54%) compared to those not reporting URTI (31%) (Nieman et al., 2006b). It is doubtful, however, that sIgA output alone can be used to predict URTI at the individual athlete level. If a 50% decrease in sIgA secretion rate is used as a

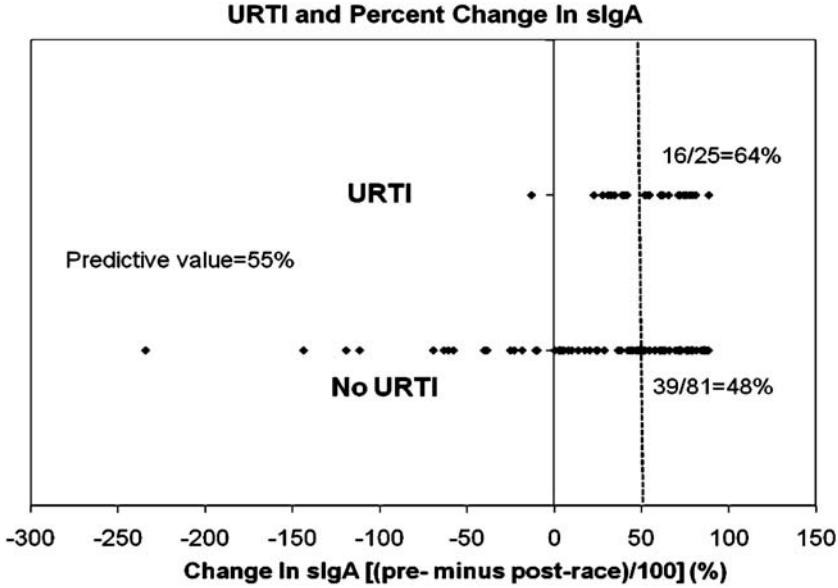


Figure 3. Scatterplot comparison of the percentage change in salivary IgA output in athletes finishing the 160-km WSER and then reporting URTI or no URTI during the ensuring 2-week period

threshold level indicating increased risk of post-race URTI, about half of non-URTI subjects would have been classified at risk (false positives) compared to about two-thirds of those reporting URTI (true positives), with an overall predictive value of 55% (Figure 3). These proportions indicate that sIgA output is more useful at the group compared to the individual level, and that other factors need to be discovered and combined with sIgA before URTI risk can be predicted for individual athletes. In this group of 350 WSER athletes, however, none of the demographic, training, or immune factors differed between URTI and non-URTI groups, indicating that the search for other predictive factors may be complex.

PLASMA CYTOKINE LEVELS

Plasma concentrations of pro- and anti-inflammatory cytokines increase during prolonged and heavy exertion including interleukin (IL)-6, IL-10, IL-8, IL-1ra, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 beta (MIP-1β), tumor necrosis factor-alpha (TNF-α), and macrophage migration inhibitory factor (MIF) (Nieman et al., 2003a, 2006a). Primary signaling mechanisms for cytokine gene expression during exercise are poorly understood, but data suggest that nitric oxide production is one key regulator (Steensberg et al., 2007; Suzuki et al., 1999). Other potential

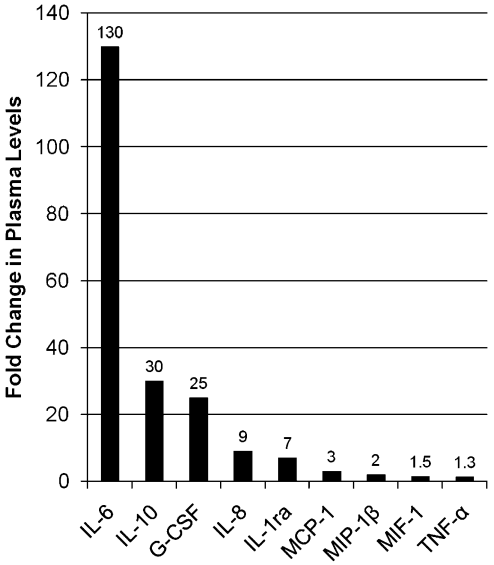


Figure 4. Pre-to-post WSER fold changes in plasma cytokine levels

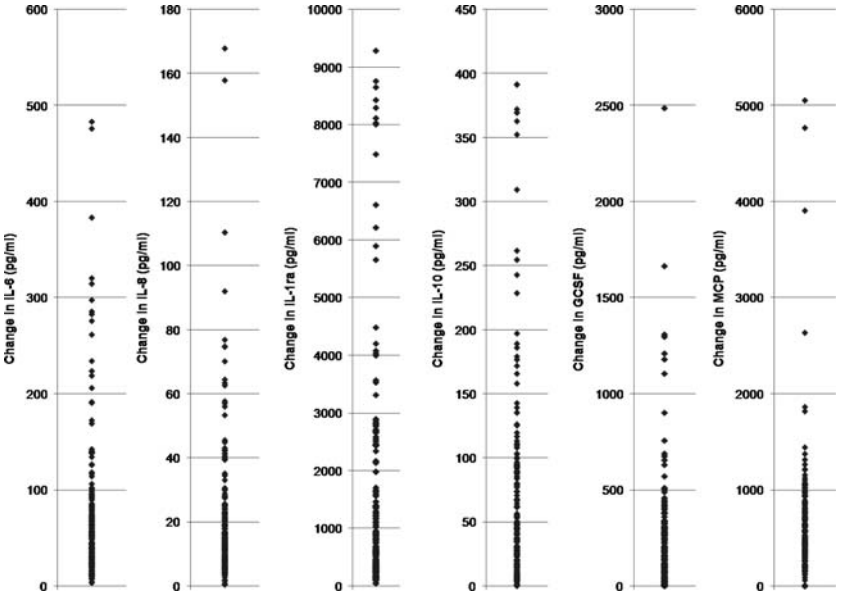


Figure 5. Scatterplots of change in plasma levels of six cytokines in ultramarathon runners competing in the 160-km WSER

triggers of cytokine release during extreme exercise include leakage of endoxtoxins (lipopolysaccharide or LPS) from the intestines, elevation in catecholamines and cortisol, high core body temperature, glycogen deficiency and other metabolic demands, muscle damage and inflammation, and oxidative stress (Suzuki et al., 1999; Nieman et al., 2006a).

At the 160-km WSER, the greatest fold-increase was experienced pre-to-post race for IL-6 (∼130-fold) followed by IL-10 (30-fold), granulocyte colony-stimulating factor (G-CSF) (25-fold), IL-8 (9-fold), IL-1ra (7-fold), monocyte chemotactic protein 1 (MCP-1) (3-fold), macrophage inflammatory protein 1 beta (MIP-1β) (2-fold), macrophage migration inhibitory factor 1 (MIF-1) (1.5-fold), and tumor necrosis factor alpha (TNF-α) (1.3-fold) (Nieman et al., 2003b, 2005, 2006a, c, 2007) (Figure 4). However, as shown in Figure 5, there was a large individual-to-individual variation in the change of plasma cytokine levels pre-to-post WSER. No difference in plasma cytokine changes was measured between male and female runners.

PREDICTORS OF VARIANCE IN PLASMA CYTOKINE CHANGES

This large variation in plasma cytokine response to running 160-km was unexpected. Ultra marathon competition is associated with significant muscle cell damage (Kim et al., 2007). Additional analysis of the entire dataset revealed that athletes with the greatest degree of muscle damage and inflammation (as measured by serum creatine kinase, CK, and C-reactive protein, CRP, respectively) experienced the highest post-race plasma levels for most of these cytokines (Nieman et al., 2005, 2006c). Figure 6 shows the large increases in CK (a) and CRP (b) that occurred during the WSER, and Figure 7 shows the scatterplot relationships of the change in IL-6 with CK (a) and G-CSF with CRP (b). During the week following the WSER, self-reported delayed onset of muscle soreness (DOMS) also showed modest positive correlations with many of the cytokines changes experienced during the race (Nieman et al., 2005, 2006c).

Contrary to expectations, the oxidative stress experienced by the WSER athletes was modest (Figure 8a) and poorly correlated with cytokine, CK, and CRP changes (McAnulty et al., 2007a, b; Quindry et al., 2008). Figure 8b shows the low correlation between change in plasma IL-6 and F2-isoprostanes ($r = –0.010$, $P = 0.918$). Small but significant correlations between changes in plasma F2-isoprostanes and IL-10 ($r = 0.21$, $P = 0.024$), MCP ($r = 0.31$, $P = 0.003$), and IL-1ra ($r = 0.18$, $P = 0.045$) were measured. In general, however, the large variance in plasma cytokine levels had little relationship to oxidative stress. Acute physical activity is typically marked by an identifiable oxidative stress within the blood plasma, and is accompanied by changes in blood redox status (Powers et al., 2004; Quindry et al., 2008). However, the endogenous anti-oxidant enzyme defenses of the WSER athletes appear capable of keeping oxidative stress at low levels during 160-km of race competition through mountainous terrain and undue environmental stress.

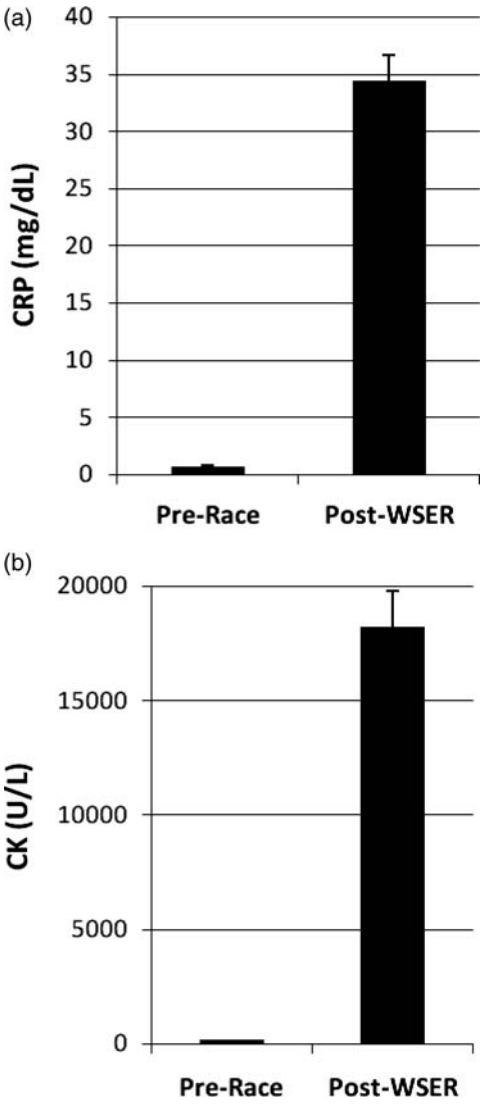


Figure 6. Pre- and post-WSER serum levels of C-reactive protein (CRP) (A) and creatine kinase (CK) (B)

Exercise-induced increases in cytokines are produced by multiple cell types both within and outside the immune system (Frydelund-Larsen et al., 2007; Keller et al., 2001; Nielsen and Pedersen, 2008). Several laboratory studies indicate that IL-6, IL-8, IL-1β, and TNF-α mRNA content is increased within post-exercise muscle biopsy samples, with the greatest fold increases measured for IL-6 and IL-8 mRNA (Nieman et al., 2003a). Laboratory studies also indicate that blood leukocytes may

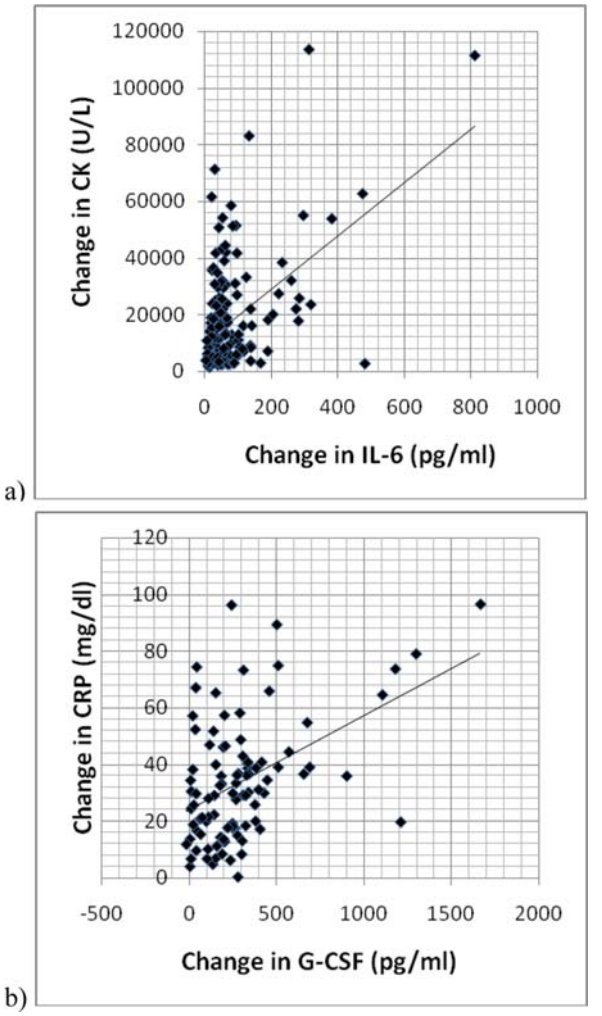
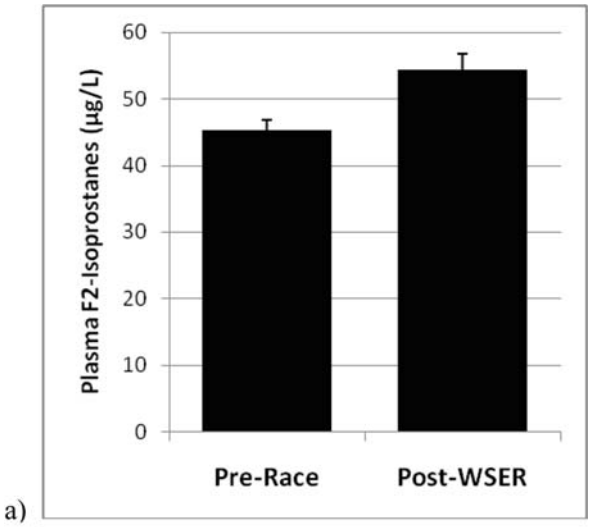


Figure 7. Scatterplots of change in plasma IL-6 with serum CK (A), and change in plasma G-CSF and serum CRP (B)

provide produce IL-8, IL-10, and IL-1ra during sustained exercise (Nieman, 2006a). Figure 9 shows that blood leukocyte IL-10 mRNA increased to high levels during the race in contrast to a sharp decrease in leukocyte IL-8 mRNA (Nieman et al., 2007). The marked decrease in leukocyte IL-8 and increase in IL-10 mRNA expression are notable findings that differ from the mild increases measured in the laboratory setting, and suggest an attempt by the immune system to limit polymorphonuclear cell adherence, degranulation, respiratory burst activity, and inflammation when the athlete is already experiencing significant muscle cell damage (Nieman et al., 2006a, 2007).



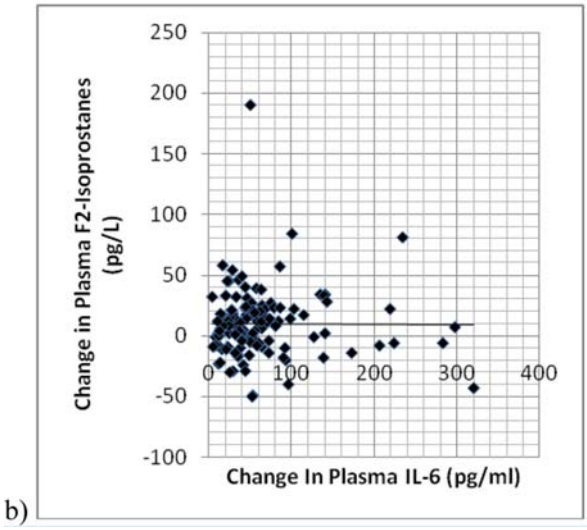


Figure 8. Pre- and post-WSER plasma levels of F2-isoprostanes (A), and scatterplot relationship between pre-to-post race change in plasma IL-6 and F2-isoprostanes (B)

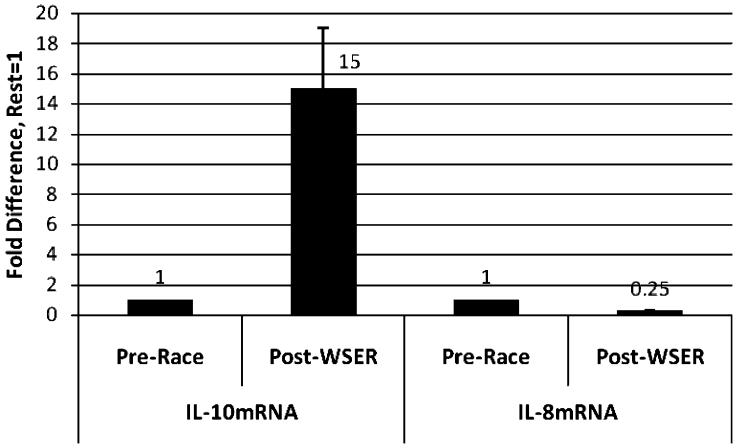


Figure 9. Fold change in blood leukocyte mRNA for IL-10 (A), and IL-8 (B)

NEGATIVE EFFECTS OF IBUPROFEN USE

Ibuprofen was the most common medication used by WSER runners, with seven in ten reporting use during the race (Nieman et al., 2005, 2006c). Ibuprofen users (600 and 1,200 mg ibuprofen the day before and during the race, respectively) and nonusers were compared pre- and post-WSER for inflammatory parameters, plasma cytokines, urine creatinine, and LPS. Ibuprofen use was associated with 25–88% higher plasma levels of seven cytokines, and significant elevations in blood neutrophils counts and serum CRP (Figure 10a), urine F2-isoprostanes (Figure 10b), alanine and asparate aminotransferase (ALT, AST), and blood urea nitrogen (BUN) (McAnulty et al., 2007a, b; Nieman et al., 2005, 2006c). Pre- and post-race plasma LPS combined was 106% higher in the ibuprofen compared to control athletes, and was positively correlated with CRP, cortisol, and three of eight cytokines measured in this study. No differences in race time, ratings of perceived exertion (RPE), gastrointestinal discomfort, muscle damage, or perceptions of muscle soreness were found between ibuprofen and control groups.

Significant increases in plasma LPS have been reported in athletes following prolonged exercise in some studies (Bosenberg et al., 1988; Brock-Utne et al., 1988), but in most studies increases are small or absent (Camus et al., 1997, 1998; Jeukendrup et al., 2000). When it does occur, exercise-induced endotoxemia has been hypothesized to be related to splanchnic ischemia and hyperthermia (Lambert, 2004). Cortisol tended to increase more in the ibuprofen compared to control group, and was correlated with change in six of eight cytokines. Thus group differences in plasma cytokine levels may have been partly related to cortisol influences. Farquhar et al. (1999) have shown that ibuprofen ingestion has a small but significant effect in decreasing glomerular filtration rate during exercise. Thus clearance of plasma

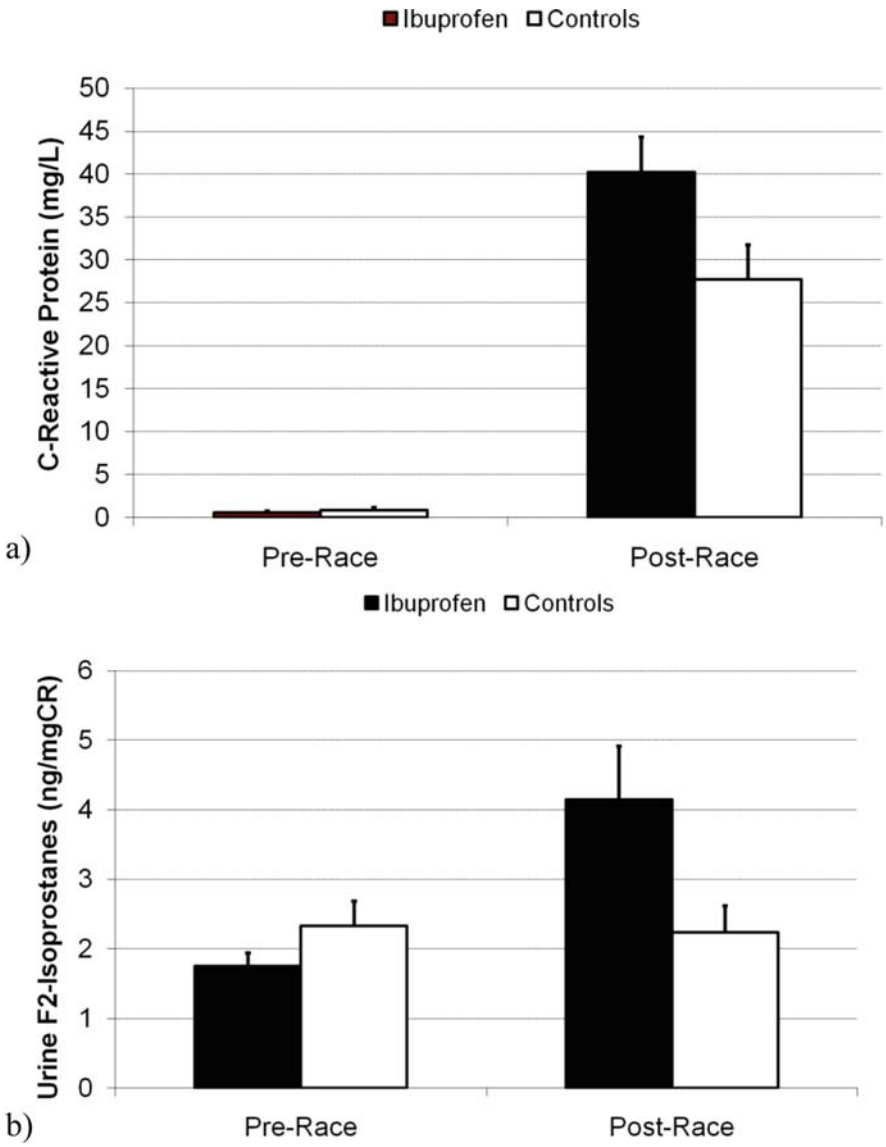


Figure 10. Comparison of pre- and post-race levels of serum CRP (A), and urine F2-isoprostanes (B) in ibuprofen users and non-ibuprofen users (controls)

cytokines by the kidney may have been decreased in athletes using ibuprofen, as supported by the urine creatinine and BUN data. Post-race serum ALT and AST levels were significantly higher in athletes using ibuprofen compared to controls. These data indicate the potential for higher muscle and liver cell enzyme

release with ibuprofen use. Significant, positive relationships between CK and most cytokines measured in the ibuprofen users was probably related to several factors including elevated LPS, a tendency for higher cortisol levels, decreased kidney clearance, increased muscle and liver cell release of enzymes, and other unmeasured parameters.

Ibuprofen use did not attenuate plasma CK levels or post-race DOMS in the WSER athletes. A majority of other investigators have also reported no beneficial effect ibuprofen or other NSAIDS in alleviating muscle soreness and damage after contraction-induced muscle injury (Donnelly et al., 1990; Peterson et al., 2003; Pizza et al., 1999; Trappe et al., 2002). Thus the high prevalence of ibuprofen use by ultramarathon athletes appears to have few if any physiological or performance benefits (Nieman et al., 2005, 2006c).

CONCLUSION

The 160-km WSER is an arduous mountain race in California reserved for the top echelon of ultra marathon athletes. Over a span of five years, 350 athletes have been studied, with immunity, inflammation, and oxidative stress measures obtained from pre- and post-race blood, urine, and saliva samples. Subjects also provided URTI, training, and demographic data. Several key findings from an analysis of the entire 5-year dataset revealed the following:

1. One out of four runners reported sickness during the 2-week period after the 160-km race. Post-race values for granulocyte oxidative burst activity were reduced 50% compared to pre-race. Of all outcome measures, only low post-race sIgA secretion rate was significantly linked to URTI. However, the predictive value was too low to be of use at the individual level.
2. The variance in plasma cytokine changes during the race was large between WSER finishers, with no male-to-female differences. The cytokine profile measured indicates a strong anti-inflammatory response.
3. The most important link to the level of plasma cytokine perturbations was muscle damage and inflammation. Athletes with the greatest serum CK and CRP levels experienced the highest post-race plasma cytokine levels. Unexpectedly, cytokine variance was unrelated to oxidative stress. Post-race blood leukocyte mRNA expression for IL-10 was unusually high in comparison to very low values for IL-8 reflecting the immune system's effort to dampen inflammation.
4. Despite the widespread use of ibuprofen by WSER athletes, no beneficial effects were measured for reducing RPE, muscle damage, or DOMS. To the contrary, ibuprofen use was linked to mild endotoxemia, kidney dysfunction, and systemic inflammation.

In general, the WSER data indicate that competitors experience significant immune system stress, reflecting the physiological trauma experienced by the athletes. The immune and cytokine perturbations measured in ultra marathon runners

at the WSER are comparable to those measured in marathon runners after running 42.2-km marathon races, but the long duration of the race means that the physiologic and immune stress is sustained for 5–10 times longer. Some of WSER athletes have competed in more than 100 ultra marathon races, and the long-term impact on health and disease remains to be determined. To reduce the pain of running 160-km in the Sierra Nevadas, the majority of WSER athletes use ibuprofen. However, every indication is that ibuprofen amplifies inflammation and oxidative stress while providing no relief from exercise effort or muscle damage and soreness.

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CHAPTER 17

HSP, EXERCISE AND SKELETAL MUSCLE

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Abstract: Skeletal muscle, which represents about 40% of the body mass in man, is an integrated organ, which possesses an extensive blood supply and innervations to support groups of muscles and muscle fibers (motor units) which must respond to a wide range of activities. Over the lifespan, skeletal muscle is forced to adapt to a variety of influences including growth and development, aging, hormonal influences, and changes in activity level. In this regard, skeletal muscle expresses the evolutionarily conserved group of proteins known as heat shock proteins (HSP) which may be critical to this adaptive process. Indeed the induction of HSP by the physiologically relevant stimulus of exercise may be harnessed as a means of protecting skeletal muscle and it's components against a variety of insults. These proteins may also be important in chaperoning muscle remodeling. The following review addresses these issues while simultaneously revealing the paucity of information available on this subject in such an important organ

Keywords: Vasculature; motor nerves; intracellular signaling; adaptation; ischemia/reperfusion, hypertrophy; atrophy

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DNP, 2,4-dinitrophenol; ERK, extracellular signal-regulated kinases; GS, glycogen synthase; HSE, heat shock element; HSF1, heat shock factor 1; HSP, heat shock proteins; Hsp70, inducible seventy kilo-dalton heat shock protein; I/R, ischemia/reperfusion; NFATc1, nuclear factor of activated T-cells; PKA, protein kinaseA; ROS, reactive oxygen species

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INTRODUCTION

All organisms possess means to protect themselves from predators, whether it is the passive protection against coyotes provided by the quills of a porcupine or the active protection against hyenas provided by the teeth and claws of a lion. Individual cells also rely on a redundant series of mechanisms to protect against insults faced on a regular basis. These defenses range from the passive presence of a number of defensive antioxidant systems (Powers and Lennon 1999) to the aggressive release of cytokines which can lead to the destruction of dangerous invaders (Petersen and Pedersen 2005). Heat shock proteins (HSP) represent a cellular protective system that arose early in evolution, and has subsequently evolved to protect whole organs and indeed organisms (Feder and Hofmann 1999). There are a number of distinct HSP, most commonly classed according to molecular weight, which operate in separate cellular compartments with the primary responsibility of maintaining the fidelity of cellular proteins. Indeed, their actions range from defensive roles in the maintenance of appropriate protein conformation (Welch 1991) to offensive roles in the activation of immune responses (Campisi et al. 2003). The purpose of this chapter is to examine the role of these molecular defenders in skeletal muscle, particularly with regard to the adaptive response to exercise. Recent reviews have described the response of the HSP to both acute exercise and exercise training in some detail (Noble et al. 2008; Whitham and Fortes 2008), hence the majority of this chapter will be devoted to the potential biological roles of these proteins and questions related to their activation.

SKELETAL MUSCLE, HSP AND ADAPTATION TO EXERCISE

Striated muscle, which represents about 40% of the body mass in man, is a complex organ which possesses an extensive blood supply and innervations to support groups of muscles and muscle fibres (motor units) which must respond to a wide range of activities. The whole is encased in and connected to the skeleton by a complicated system of connective tissue which is not only responsible for force transmission but has many signaling roles as well (Kjaer 2004). The degree of muscle activity varies between individuals, as well as between different muscles and within individual muscle fibres themselves. Indeed, motor nerves to skeletal muscles in rats may fire as frequently as 10 times per second for muscle fibres associated with postural maintenance, to as infrequently as 1–2 bursts per week for those fibres involved in rapid high intensity movement (Hennig and Lomo 1985). In addition, skeletal muscle is forced to quickly adapt throughout the lifespan to a variety of influences including growth and development, aging, hormonal influences and changes in activity level (Lee et al. 2007). As with other tissues, skeletal muscle expresses a full range of HSP that are critical in enabling adaptation to this ever changing environment. These include the constitutive and inducible members of the 70 kDa HSP family, Hsc70 and Hsp70, respectively, as well as the mitochondrial chaperones Hsp60 and Hsp75. Hsp90, Hsp25/27 and the low-molecular weight chaperones such as Hsp20,

the crystallins and heme oxygenase, the connective tissue chaperone, Hsp47, and a number of additional HSP and numerous accessory proteins that are also present in skeletal muscle. Rather than detail the general roles and cellular locations of individual HSP here, the reader is referred to the following reviews (Noble 2002; Noble et al. 2008; Welch 1991).

FIBRE TYPE DIFFERENCES IN HSP – BASAL STATE

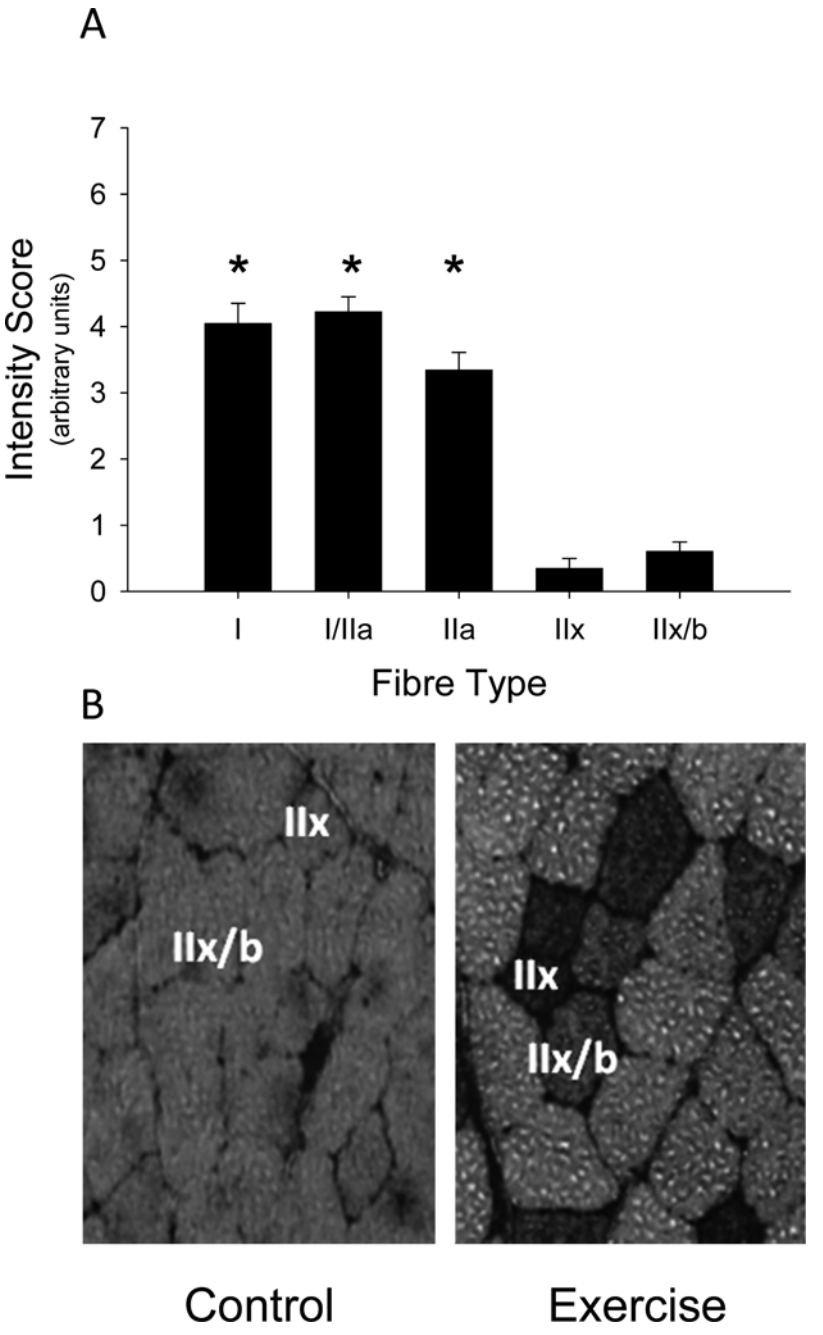
Contractile activity has the potential to introduce a number of perturbations to skeletal muscle. These include increased temperature, altered metabolic milieu and frank muscle damage. Hence, it is not surprising that this tissue exhibits significant levels of HSP, even in the basal state (Noble 2002). However, as noted above, skeletal muscle exhibits a mix of fibre types which have been referred to as types I, IIa, IIx (d) and IIb (Table 1). These fibre types represent a continuum of contractile and metabolic properties. Hence, type IIb fibres are the least likely to be recruited and exhibit the lowest oxidative capacity, greatest maximal shortening velocity, maximal power and ATP use, while type I fibres demonstrate the other end of this recruitment metabolic capacity continuum (Bottinelli and Reggiani 2000). Hybrid fibres fill the gaps between pure fibres according to a "nearest-neighbour" rule such that IIa/x (d) fibres exist between pure IIa and IIx(d) fibres etc. (Pette and Staron 2001). Under basal conditions, more tonically active postural muscles and muscle fibres (type I and IIa) tend to have greater levels of HSP (Noble 2002) (Figure 1a), and it has been argued that this may be due to increased loading (Locke et al. 1991) and particularly the increased oxidative stress experienced by activated muscle (Fischer et al. 2006; Khassaf et al. 2003). While such fibre type differences are evident in rodent models (O'Neill et al. 2006; Ogata et al. 2003) they are less apparent in humans, probably because the latter have muscle fibres which almost exclusively express the Type I or IIa myosin known to be associated with elevated HSP content (Huey et al. 2004; O'Neill et al. 2006; Tupling et al. 2007). Indeed, under a broad variety of conditions, which alter muscle load and phenotype such as altered thyroid hormone levels (Locke et al. 1994; O'Neill and Noble 2004; Ogata et al. 2005) muscle hypertrophy (O'Neill et al. 2006; Oishi et al. 2004) and development (O'Neill and Noble 2004; Ogata et al. 2003), HSP content appears more related to myosin phenotype than activity patterns alone, with high constitutive expression of Hsp70 in Type I and IIa fibres but low expression in Type IIx and IIb fibres (O'Neill et al. 2006). Similar observations have been made for other HSP (Golenhofen et al. 2004; Noble 2002) although strenuous, acute or chronic exercise can increase these HSP in all fibre types (Figure 1b).

It is unclear what specific role HSP play in slower, oxidative (Type I or IIa) muscle fibre types or why they exhibit increased expression in the unstressed basal state. It could be that under basal conditions, high constitutive expression of HSP has more to do with their role in cell signaling and ultimate regulation of transcription than in the need for increased protection. In this regard, the expression of slower, more oxidative

Table 1. Skeletal muscle properties and HSP expression

Muscle type	Primary metabolic pathway	Muscle contractile properties	Fatigue resistance	Mitochondrial density	Capillary density	Function	Example	HSF1 expression	Basal HSP expression
Type Ia	Oxidative	Slow	High	High	High	Postural, chronically activated	Soleus muscle	High	High
Type IIa	Oxidative/ glycolytic	Intermediate	Moderate	Moderate	Moderate	Everyday activity	Red vastus	Moderate	High/moderate
Type IIx/d	Oxidative/ glycolytic	Intermediate	Moderate	Moderate	Moderate	Power	Plantaris	Moderate	Moderate
Type IIb	Glycolytic	Fast	Low	Low	Low	Power	White vastus	Low	Low

Although these general classifications hold, they may be misleading since, for example, capillary density is not a function of the muscle fiber type per se, but rather a property of muscle high in the that particular fiber type. In addition, although example muscles are composed largely of the fiber type mentioned, they do have a mixed fiber type composition.


A
Intensity Score
(arbitrary units)
7
6
5
4
3
2
1
0
*
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I
I/IIa
IIa
IIx
IIx/b
Fibre Type
B
IIx
IIx/b
IIx
IIx/b
Control
Exercise

Figure 1.

muscle fibre-specific phenotypes has been proposed to be activated by the calcium, calmodulin-dependent serine/threonine protein phosphatase, calcineurin (Allen and Leinwand 2002; Dunn et al. 2001; Long and Zierath 2008). In skeletal muscle, one of the primary targets of calcineurin, an isoform of the calcineurin-nuclear factor of activated T-cells (NFATc1), is found endogenously only in Type I and IIa muscle fibres (Mutungi 2008), the same endogenous distribution as that observed for most HSP and Hsp70 in particular. Importantly, calcineurin activity may be regulated by Hsp70 and Hsp90 (Someren et al. 1999; Tumlin et al. 1997). Exercise is associated with increased phosphorylation of Hsp70 (Hernando and Manso 1997; Melling et al. 2008) and this phosphorylation results in maximal activation of calcineurin (Lakshmikuttyamma et al. 2004). Indeed, exercise-induced modulation of calcineurin activity parallels the changes in muscle fibre type resulting from exercise (Grondard et al. 2008). This role for HSP in calcineurin activation could also account for the observation that a decrease in Hsp70 and Hsp90 in muscle from animals subjected to clenbuterol, was associated with a decrease in slow oxidative muscle fibres despite an increase in calcineurin protein content (Oishi et al. 2004). Hence, increased HSP content, whether present under basal conditions or in response to exercise, could be important in the remodelling and maintenance of a slower more oxidative muscle phenotype in addition to offering protection against insult (see later in this chapter). It would be interesting to determine whether the same activating effects of Hsp70 on calcineurin are important in the extracellular roles that Hsp70 plays with the immune response (Campisi et al. 2003; Campisi and Fleshner 2003).

One unanswered question is the mechanism by which increased constitutive expression of certain HSP are maintained in slower more oxidative muscle fibres. The pre-existing heat shock transcription factor, HSF1, appears to be the primary factor involved in the expression of HSP in skeletal muscle (see below), yet there does not appear to be increased basal activation of HSF1 in either muscles (Locke and Tanguay 1996) or muscle fibres (unpublished observations) expressing Type I or IIa myosin compared to the faster phenotypes. Others have noted that in most tissues, Hsp70 is encoded for by several transcripts of which *hsp70.1* to *hsp70.2* are most abundant and have suggested that differences in cellular Hsp70 content could be a consequence of preferential transcription of one of these species of mRNA (Angeletti et al. 1996). To investigate whether this is the case, mRNA was extracted from muscles exhibiting different fiber type proportions and subsequently amplified

Figure 1. Fiber type specific expression of Hsp70 in skeletal muscle at rest and following exercise. (**a**) Relative differences in Hsp70 in muscle fibers expressing various myosin isoforms in the rat plantaris. Note the significantly greater ($*p<0.05$) expression of Hsp70 in fibers expressing type I or IIa myosin versus the faster contracting less oxidative IIx and IIb myosin. (**b**) Muscle cross-sections from the white portion of rat vastus muscle (which expresses only IIx and IIb myosins) stained for Hsp70. Under control conditions there is little detectable Hsp70 but 24 h following 1 h of strenuous exercise, Hsp70 is increased in selected fibers of the exercised muscle. It is likely that the increase is related to motor unit recruitment.

and probed for *hsp70.1* to *hsp70.2* transcripts using real-time PCR. The results indicated that although the ratio of *hsp70.1* to *hsp70.2* was greater in those muscles expressing greater basal levels of Hsp70 (Figure 2a) both transcripts were present in similar absolute amounts in muscles of differing fibre composition and thus selective transcription of a particular mRNA likely could not account for the observed basal differences in HSP content (Figure 2b). It has also been suggested that in addition to HSF1 other related heat shock factors, such as HSF2, may be more important in muscle development and restructuring (McArdle et al. 2006). In this regard, Locke (2007) examined the involvement of both HSF1 and HSF2 in the induction of HSP70 and Hsp25 that occur in the remodeling hypertrophying muscle, and was unable to demonstrate that either was important in this process. Hence, the underlying mechanisms responsible for basal fibre differences in the expression of HSP have yet to be fully elucidated.

EXERCISE INDUCED CHANGES IN HSP

As stated above, skeletal muscle represents a complex organ comprised of several types of tissues. In addition to contractile units and the connective tissue necessary to maintain muscle structural integrity, muscle contains blood vessels, which direct and carry blood flow to the working muscle fibres, as well as nerves necessary for movement and feedback. When considering the functional implications of HSP induction following exercise it is appropriate to examine skeletal muscle with respect to each of these components.

Skeletal Muscle Tissue

Acute exercise represents an unaccustomed stress which is accompanied by increased temperature, metabolic disturbance and potential muscle damage. As a result, acute exercise can cause an increase in skeletal muscle HSP (Noble 2002). Of course, this response is variable with exercise type, intensity and duration (Milne and Noble 2002; Morton et al. 2006; Paulsen et al. 2007) as well as with prior training status (Gjovaag and Dahl 2006; Morton et al. 2008; Thompson et al. 2002). In general, non-damaging isometric type exercise leads to elevated levels of Hsp70 and Hsp60 but less of a response in the small HSP (Koh and Escobedo 2004; Locke et al. 1990; Morton et al. 2006; Tupling et al. 2007). In exercising muscle, the metabolic demands are high necessitating increased fuel and gas transport and, likely as a consequence, increased oxidative stress on many muscle organelles (Radak et al. 2008). In response to stress, Hsp70 may protect mitochondrial (Bornman et al. 1998; Ellis et al. 2000; Sammut et al. 2001) and sarcoplasmic reticulular function (Tupling et al. 2004). Although the direct link is not known, Hsp70 is also important in maintaining insulin sensitivity and ultimately glucose utilization in exercising muscle (Kurucz

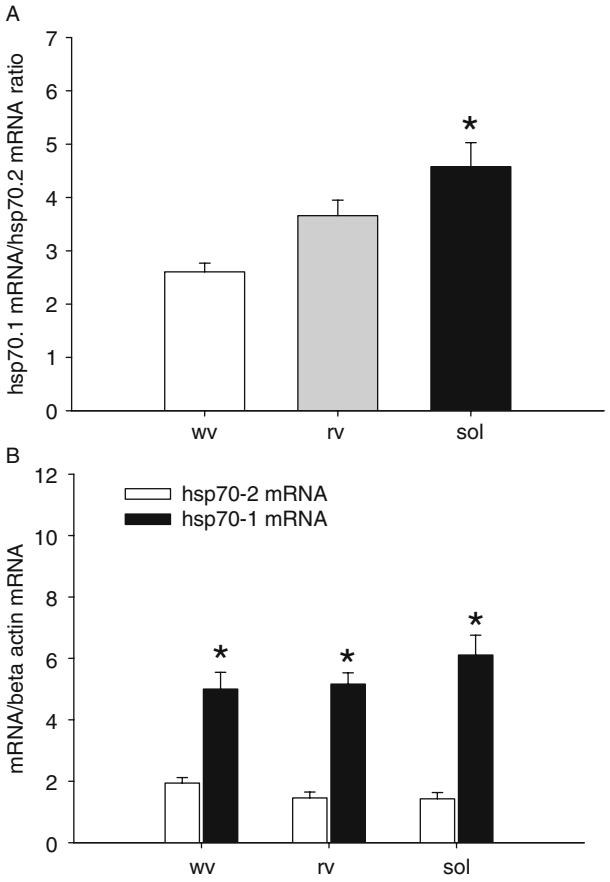

Figure 2. Hsp70 mRNA expression in skeletal muscle. (**a**) The ratio of *hsp70.1* to *hsp70.2* mRNA in skeletal muscles composed of different fiber types which exhibit varying constitutive expression of Hsp70. wv-the white portion of the vastus muscle group which is composed solely of type IIx and IIb fibers and has a low level of constitutive expression of Hsp70; rv-the red portion of the vastus muscle group which is composed primarily of type IIa fibers and has a moderate constitutive expression of Hsp70; sol-soleus muscle composed primarily of type I muscle fibers which has a high constitutive expression of Hsp70. ∗sol is significantly greater than wv (p<0.05). (**b**) The absolute amount of *Hsp70.1* and *Hsp70.2* mRNA in skeletal muscles composed of different fiber types which exhibit varying constitutive expression of Hsp70. ∗*hsp70.1* expression is significantly greater than *hsp70.2* mRNA expression in all muscles (p<0.05), however there were no absolute differences in expression of these mRNA species between muscles.

et al. 2002). Hsp60 may protect muscle by inhibiting the ubiquitination of key pro-survival molecules such as insulin-like growth factor-1 (Lai et al. 2007; Shan et al. 2003). Lastly, several HSP that are induced with exercise may suppress the inflammatory response and thereby limit muscle damage (Chen et al. 2007). However, HSP

induction cannot totally rescue the function of exhaustively exercised muscle (Nosek et al. 2000; Thomas and Noble 1999).

With acute damaging exercise (such as eccentric resistance exercise) the responses are often more dramatic. Not only are increases in Hsp70 observed, but the small HSP, Hsp25/27 and αB-crystallin are found to increase after a variety of eccentric contractions including downhill running (Feasson et al. 2002) and forced muscle extension (Koh and Escobedo 2004; Paulsen et al. 2007; Thompson et al. 2001). Although there is little information regarding the cellular localization of HSP following exercise, some evidence is emerging. For example, with eccentric damaging contractions, small HSP (αB-crystallin and Hsp25/27) are rapidly phosphorylated, reduced in oligomeric size and translocated to selected myofibrillar proteins and the cell membrane where they likely protect the cytoskeleton (Arrigo 2007; Koh and Escobedo 2004; Paulsen et al. 2007). αB-crystallin may also maintain myosin enzymatic activity in these muscles (Melkani et al. 2006) and this may partially account for relationships between small HSP and maintenance of force-generating capacity in damaged muscle (Paulsen et al. 2007). Interestingly, translocation of the small HSP was not noted in muscles undergoing less-damaging isometric contractions (Koh and Escobedo 2004). Hsp70 also appears to be induced with potentially damaging exercise (Hsu et al. 2005; Koh and Escobedo 2004; Paulsen et al. 2007; Thompson et al. 2002) and has been observed localized with the myofibrillar fraction (Paulsen et al. 2007). With few exceptions, Hsc70, the constitutive isoform of the 70 kDa family is not found to increase with exercise (Kelly et al. 1996; Murlasits et al. 2006).

With exercise training, presumably skeletal muscle has adapted to activity and the stress of each individual exercise bout is decreased. However, despite the fact that exercise training reduces the stress of individual work bouts, most HSP (including 60,70,75 and 78) tend to remain elevated in the muscle of individuals undergoing endurance non-damaging concentric contractions (Gonzalez et al. 2000; Morton et al. 2008; Noble 2002). In several tissues, however, it appears that unless an exercise training stress is maintained or increased, basal levels of most HSP return to pre-training levels or lower (Fehrenbach et al. 2000; Gonzalez et al. 2000; Liu et al. 2000). Muscles that exhibit high constitutive levels of Hsp70 tend to exhibit a reduced relative response (percent increase) in HSP to either endurance exercise (Milne and Noble 2002; Morton et al. 2008) or eccentric resistance loading (Gjovaag et al. 2006). Nonetheless, the capacity to respond to exercise in an absolute sense (protein synthesized) may be undiminished or enhanced (Gonzalez et al. 2000; Locke and Tanguay 1996; Milne and Noble 2002).

Blood Vessels

As noted above, the ability to successfully undergo locomotion is dependent upon more than the contractile function of the skeletal muscle fibres. An extensive network of blood vessels must supply nutrients to and remove end products from the working muscle as well as to help regulate the optimal working temperature. Indeed,

peak blood flow may increase by over 100-fold in exercising skeletal muscle and this increase is under exquisite control (Joyner and Wilkins 2007). The increased flow may exert both shear stress and cell stretch on the endothelium and the underlying smooth muscle that may be responsible for the induction of various HSP (Wang et al. 2007). In the short term, this induction may protect the vessels and the organs they serve against a variety of insults. For example, in the myocardium (Leger et al. 2000) and brain (Ikeda et al. 2000), heat shock is associated with increased levels of several HSP in the vasculature, an increase that has been linked with enhanced tolerance to ischemia-reperfusion injury (Ikeda et al. 2000; Milne and Noble 2007), protection against myointimal hyperplasia and smooth muscle cell hypertrophy (Connolly et al. 2003; Denes et al. 2008; Neschis et al. 1998; Tessier et al. 2004; Zheng et al. 2006), inflammation (Chen and Currie 2006; Wong et al. 1995), maintenance of the endothelial barrier (Chatterjee et al. 2008; Hirano et al. 2004) reduced leukocyte-endothelial interaction (McCormick et al. 2003) and survival of smooth muscle cells (Johnson et al. 1995). Recently, exercise has also been demonstrated to induce HSP in the vasculature of both skeletal (Tarricone et al. 2008) and cardiac (unpublished observations) muscle. Indeed, in the myocardium, significant increases of Hsp70 observed, post-heat shock treatment (Amrani et al. 1998; Leger et al. 2000) are almost exclusively limited to the vasculature. Hence, as with heat shock (Currie et al. 1988), the protection afforded by exercise against ischemia-reperfusion injury in the heart (Paroo et al. 2002) could be partially a consequence of this vascular expression of HSP. Even in skeletal muscle, where only certain muscle fibres exhibit enhanced expression of HSP (Noble 2002; Tupling et al. 2007), the vasculature demonstrates extensive upregulation of Hsp70 and 25/27. Although observed in both the endothelium and smooth muscle compartments of the vasculature, the greatest expression is associated with smooth muscle (unpublished observations). It is likely that the exercised-induced increase of these proteins protects the vasculature and therefore the muscle itself in a similar fashion to that afforded by heat shock as described above.

Repetitive exercise training ultimately results in arteriogenesis (vessel enlargement) or angiogenesis (capillary formation) as a means to enhance the exchange of nutrients (Prior et al. 2004). HSP may be important in this regard. Regardless of whether capillaries divide in response to the increased blood flow (luminal shear stress) accompanying exercise, or sprout due to stretch of the surrounding contractile tissue (abluminal signals) (Egginton et al. 2001), HSP increase in endothelial cells (Wang et al. 2007; Xu and Wick 1996) and these HSP may be involved in many of the processes leading to growth of the vascular system. For example, suppression of Hsp90 inhibits angiogenesis (Milkiewicz et al. 2007). Moreover, endothelial and smooth muscle cell motility and proliferation (an important part of stretch-induced angiogenesis) are impaired if Hsp25/27 is inhibited (Hedges et al. 1999; Keezer et al. 2003) and indeed Hsp70 appears to be important in this proliferation (Martinez et al. 2006). Lastly, Hsp47 may be critical in chaperoning the remodeling of the collagen associated with arteriogenesis or angiogenesis (Sluijter et al. 2004). Hence, the HSP that are induced in endothelial and

smooth muscle cells in response to exercise (Tarricone et al. 2008) may play an important role in the training adaptations that occur in skeletal muscle while simultaneously protecting the vasculature and organs from the physical and metabolic consequences of increased blood flow. Moreover, it is interesting to note that muscles consisting of primarily Type I or IIa muscle fibres also possess a high capillary density. Consequently, part of the enhanced expression of HSP in these types of muscle may additionally be related to vascular specific expression. This raises an important methodological consideration in that simply analyzing the total homogenate of skeletal muscle or any tissue for that matter is no longer enough and hence, information about compartmental and/or cell-specific expression of HSP or other gene products is needed to adequately identify potential roles of these proteins.

Motor Nerves

The third major component of integrated muscle is the motor nerves which activate individual motor units and ultimately muscle fibres during exercise. There is little evidence that declines in function of either the central or peripheral nerves normally limit the ability to exercise in young healthy individuals, although there is the possibility that central activation and processes at the neuromuscular junction may contribute to fatigue (Gardiner 2001). However, motor neurons innervating muscle do adapt to exercise and exercise training and the loss thereof (Gardiner et al. 2005) and during exercise they likely undergo many of the same passive stresses, such as increased metabolic rate and temperature faced by the muscle fibres they innervate (Morton et al. 2007). Although there is a paucity of data, it is reasonable to assume that under these circumstances, HSP may be involved in either adaptive or functional changes. For example, when rat sciatic nerve is locally heated at 43°C for 30 min and then subsequently heated to 45°C 24 h later, degenerative changes which are normally associated with a complete loss of motor function are not observed and it is likely due to protection of vascular endothelium and myelin cells (Hoogeveen et al. 1992, 1993). Hsp25/27 may also be increased in motor nerves in response to heating (Murashov et al. 1998) and overexpression of Hsp25/27 rescues motor nerves following injury and leads to a recovery of muscle contractile function (Sharp et al. 2006). Part of the protective effect of heat induced changes in motor nerves could be related to a positive modulation of ion channels. Regulation of synaptic calcium levels by increased Hsp70 in the nerve terminals of motor neurons (either as a result of thermal pre-conditioning or transgenic manipulation) maintains synaptic transmission under heat stress (Klose et al. 2008). Hsp70 may also act to protect mitochondrial potassium channels (Shinohara et al. 2007) and thereby maintain mitochondrial respiratory function and nerve conduction under stressful conditions. Various HSP are also involved in the maturation and maintenance of other potassium channels which are critical for the proper function of many excitable tissues (Ficker et al. 2003; Ruete et al. 2008). Interestingly, as discussed in other chapters in this book, HSP

are released into the circulation in response to exercise and indeed these circulating HSP may have beneficial effects with regard to neuronal function in skeletal muscle (Robinson et al. 2005, 2008). Hence, from a number of models, HSP are known to be neuroprotective (Mattson et al. 2004) but there is limited direct data on how they might do so in response to exercise and particularly with regard to peripheral motor neurons. Since these nerves are an essential component of the skeletal muscle locomotory system, further investigation is warranted.

INTRACELLULAR SIGNALING AND EXERCISE-INDUCED ACTIVATION OF HSP EXPRESSION

At the transcriptional level, HSF1 binds to the conserved heat shock element (HSE) located in the promoter region of the HSP genes (Holmgren et al. 1981; Locke et al. 1995). Under normal conditions, HSF1 is a latent monomeric protein bound to several HSP, maintaining HSF1 in an inactive monomeric state (Abravaya et al. 1992). Following exposure to even moderate cellular stress, protein unfolding and denaturation can occur, causing HSP to preferentially bind these non-native protein products to assist in their proper refolding, thereby dissociating from HSF1 (Baler et al. 1992). Once free, HSF1 undergoes a series of complex regulatory events that include nuclear localization, oligomerization, acquisition of DNA-binding, and transcriptional expression of HSP genes (Sarge et al. 1993).

As a result of exercise, several events occur in and around skeletal muscle that are potential inducers of HSF1 activation (Figure 3). Most notably, the generation of reactive oxygen species (ROS) (Essig and Nosek 1997), changes in intracellular

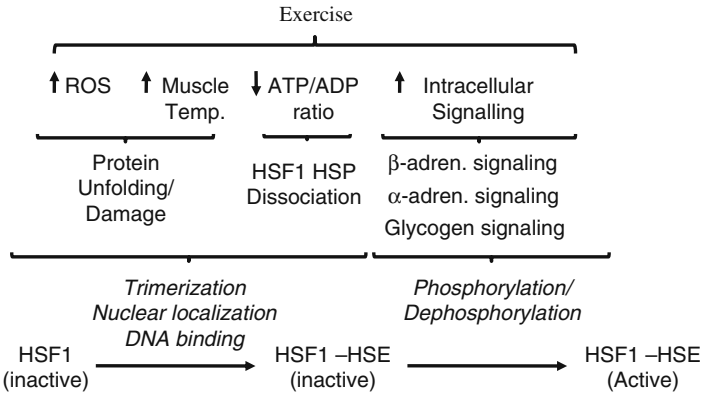

Figure 3. Schematic representation of the regulation of HSF1 transcriptional activation through intracellular signaling following exercise. Abbreviations: ROS, reactive oxygen species; Temp, temperature; Adren, adrenergic receptor; HSF1, Heat Shock Transcription Factor; HSE, Heat Shock Element located in the heat shock gene promoter region.

calcium levels (Maglara et al. 2003), and glycogen content (Febbraio et al. 2002), as well as increases in whole body and muscle temperature (Oishi et al. 2002) all occur with exercise, are dependent on exercise intensity and can cause damage to intracellular proteins leading to a heat shock response (for review see Noble 2002). While it is difficult to dissociate these factors from each other, it has been proposed that the induction of HSP expression is primarily a response to the elevated temperature and oxidative stress associated with the exercise (Salo et al. 1991). In fact, the increased temperature of isolated muscles to values comparable to those observed during exercise has resulted in enhanced expression of several HSP (Oishi et al. 2002). However, temperature-mediated elevations in HSP synthesis can also be dissociated from the typical exercise-induced expression of HSP (Morton et al. 2007) and exercise in the absence of increased temperature may lead to increased HSP expression in exercising muscle (Skidmore et al. 1995). While resolution of these discrepancies await further research, these studies indicate that while cellular oxidative stress and hyperthermia resulting from exercise are clearly capable of eliciting the heat shock response, it is more likely that a collection of factors leads to the production and accumulation of abnormal protein products and cellular vulnerability to altered homeostasis causing HSF1 activation and subsequent HSP expression.

Altered intracellular levels of high energy phosphates (ATP and ADP) are reported to elicit the transcriptional activation of HSF1 in vitro (Benjamin et al. 1992). HSP requires ADP as a cofactor for binding of peptides and their binding affinity is substantially lower in the presence of ATP (Glick 1995). Not only does ATP depletion amplify the concentration of aggregated protein products (Nguyen and Bensaude 1994), a change in ATP/ADP may increase free HSP bound to peptides and decrease HSF1-HSP interactions (Moseley 2000). Indeed, moderate decreases in intracellular ATP levels in the heart correlate with increased activation of HSF1 (Chang et al. 2001). While more work is needed to determine the role, if any, of ATP/ADP levels in HSP expression in skeletal muscle during exercise, it certainly deserves further investigation as this may represent an important mechanism that regulates the activation of HSF1 independent of the production of abnormal protein products. Even further, depletion of ATP to critical levels, which is unlikely in the heart but which may occur in skeletal muscle following strenuous exercise, has been shown to hinder, rather than activate HSF1. Given that skeletal muscle can show fibre type specificity in the activation of the heat shock response, this may explain why muscle comprised of certain fibre types show differences in the induction of this response following exercise (Liu et al. 2006).

While differences exist in skeletal muscle fibre-type specific HSP expression, HSF1 binding is a necessary event for the induction of HSP expression. Several studies have shown that while in the quiescent muscle there is little HSF1 bound to the HSP gene, upon exposure to a stressful event, HSF1 localizes to the nucleus and binds to the HSE (Melling et al. 2004; Najemnikova et al. 2007). However, reports have indicated that HSF1-HSE DNA-binding alone may not be sufficient to induce transcriptional activity of HSF1, suggesting an additional event in the regulation of HSF1 transcriptional activation (Jurivich et al. 1992). The available evidence would

strongly support the role of post-translational modifications of HSF1 in regulating its transcriptional activation. In several tissues including the heart (Melling et al. 2004), as well as other organs (Chan et al. 2003; Heydari et al. 2000) HSF1 has been shown to change its phosphorylative status upon transcriptional activation (Xia and Voellmy 1997). Ultimately, post-translational modification of HSF1 may serve as a secondary mechanism that regulates HSF1 transcriptional activation, controlling whether a muscle cell reacts to stressful stimuli with HSP synthesis. Indeed, the brain and other neuronal tissues, demonstrate selective expression of HSP genes even following hyperthermic treatment (Flanagan et al. 1995; Tonkiss and Calderwood 2005). A limited activation in these tissues is not due to lower levels of transcriptional material but rather is believed to be a result of a higher activational threshold (Batulan et al. 2003; Tonkiss and Calderwood 2005). Motor neuronal cells overexpressing HSF1 demonstrate limited expression of HSP following heat treatment, but HSP expression is elicited in these cells with deletion of the HSF1 regulatory domain (deletion of amino acids 202-316), suggesting the importance of regulatory events (Batulan et al. 2003).

The search for the intracellular signaling pathway responsible for phosphorylation of HSF1 has generated equivocal results. At least eight different signaling-related protein kinases have been shown to phosphorylate the HSF1 molecule in vitro and regulate its transcriptional activity (Chu et al. 1996; Dai et al. 2000; Fritsch and Wu 1999; Holmberg et al. 2001; Hung et al. 1998; Kim et al. 1997; Melling et al. 2007; Soncin et al. 2003). While some redundancy is to be expected with such a conserved response, a plausible explanation as to why a specific regulatory kinase has not been identified is that regulation of HSF1 is both stress and tissue specific. While the majority of studies have employed cell models, an important role for adrenergic signaling pathways (α- and β-) in the expression of HSP in vivo is emerging (Johnson et al. 2005; Melling et al. 2007; Meng et al. 1996; Paroo and Noble 1999). During exercise, stimulation of both of α- and β- adrenergic receptors (Francis et al. 1982), leads to increased activity of their downstream protein kinases PKA and PKC, respectively (Carson and Korzick 2003; Melling et al. 2004). Cell culture models have suggested that both PKA and PKC, directly phosphorylate and subsequently regulate the transcriptional activation of HSF1 (Ohnishi et al. 1999).

In the myocardium, following exercise, β-adrenergic activation of PKA is necessary for the phosphorylation and subsequent inhibition of a key kinase (ERK1/2) that represses HSF1 (Melling et al. 2006). ERK1/2-mediated phosphorylation of HSF1 is a priming step for a cascade of events leading to the binding of a group of accessory proteins that can sequester HSF1 and constrain its transcriptional ability (Wang et al. 2003). Interestingly, activation of ERK1/2 does not alter the nuclear localization and binding ability of HSF1 despite transcriptional repression, suggesting phosphorylation as a regulator of HSF1 independent of binding competence. While it is yet to be determined if the same signaling systems in the heart regulates the transcriptional activation of HSF1 in skeletal muscle, Paroo and Noble (1999) demonstrated that activation of the β-adrenergic signaling pathway potentiated the exercise-mediated expression of Hsp70 in both heart and skeletal muscle.

Contrary to PKA, the role of PKC and α-adrenergic receptor mediated signaling in the transcriptional regulation of HSF1 is less clear. Direct inhibition of PKC during exercise does not impair the translocation of HSF1 to the hsp70 gene or its transcriptional activation. Again, little is known in this regard for skeletal muscle and it would be dangerous to assume that activation mechanisms are identical between cardiac and skeletal muscle.

The α-adrenergic receptor mediated signaling pathway may play a more critical role in the induction of HSP within the walls of major vessels. Vascular smooth muscle cells (VSMCs) contained within the aorta and vena cava has been shown to elicit robust HSP expression following hypertensive stress and heat shock treatment which is directly influenced by α-adrenergic receptor activation (Udelsman et al. 1986, 1993). Similarly, tissue specific regulation of HSF1 is apparent in other tissues such as the liver that, like vasculature, contain substantially higher levels of α-adrenergic receptor (Huerta-Bahena et al. 1983). In fact, heat shock induced expression of Hsp70 and Hsp25/27 in hepatocytes isolated from the liver are not regulated by β-signaling system activation at all (Inaguma et al. 1995), but enhanced phosphorylation of HSF1 and subsequent Hsp70 mRNA (Ikeyama et al. 2001) is elicited following treatment with agents which increase PKC activation (Uchida et al. 2006; Yamanaka et al. 2003).

As mentioned above, in skeletal muscle the identification of important exercise-related signaling cascades involved in the regulation of the HSF1 and HSP expression are not well understood. Skeletal muscle contains little α-adrenergic receptor, except within the vessels of its microcirculation (Marshall 1982), and the amount of β-receptors vary depending on fibre type composition (Jensen et al. 1995). Type I muscle fibres have been reported to contain as high as three times the density and distribution of β-adrenergic receptors as Type II muscle fibres in human skeletal muscle (Martin et al. 1989). Moreover, skeletal muscle demonstrates a β-adrenergic receptor mediated potentiation of Hsp70 expression in a fibre-type specific manner (Paroo and Noble 1999), although this expression does not correlate to muscles which demonstrate the highest levels of β-adrenergic receptor density. Hence, it is difficult to ascertain a specific role for a particular signaling system in the regulation of the exercise-induced, heat shock response, as different exercise protocols result in the selective activation of converging and specific intracellular signaling pathways in skeletal muscle (Nader and Esser 2001).

It has been reported that muscle glycogen availability is a key signal regulating induction of HSP in contracting human skeletal muscle (Febbraio et al. 2002; Febbraio and Koukoulas 2000). While it is presently unclear as to the manner in which lowered glycogen levels mediate HSF1 activation, an indirect role for changes in ATP/ADP levels resulting from substrate depletion may exist (Baldwin et al. 1999; Chang et al. 2001; Sahlin et al. 1990). Selective glycogen depletion patterns occur in different muscle fibre types, depending on exercise intensity (Gollnick et al. 1974) and muscle recruitment pattern (Saltin 1981). This could possibly explain why discrepancies exist in HSP responses to exercise in muscles composed of different fibre types, and indeed between muscle fibers within the same muscle (Tupling

et al. 2007). Glycogen related signaling pathways may also regulate the phosphorylation of HSF1 during replenishment of depleted glycogen stores. Immediately following exercise, skeletal muscle fibres replenish glycogen through the dephosphorylation and activation of the primary glycogen building enzyme, glycogen synthase (GS) (Ivy and Kuo 1998). Essential to the activation of GS and recovery of muscle glycogen stores is the deactivation of GSK3α, a protein kinase responsible for the phosphorylation and inactivation of GS (Markuns et al. 1999). As a priming step for the ERK1/2-mediated phosphorylation and repression of HSF1, Gsk3α phosphorylates HSF1 on ser303 (Wang et al. 2003). Therefore, deactivation of GSK3α following exercise may cause the suppression of this HSF1 repressor kinase and subsequently induce the transcriptional activation of HSF1.

In addition to regulation of HSP genes at the transcriptional level, it is now evident that post-translational alterations are important to the induction of HSP proteins. Following exercise, Hsp70, has been shown to exist in three different phosphorylative forms in several tissues including the heart (Melling et al. 2008), liver (Gonzalez and Manso 2004), and several skeletal muscles of different fibre type composition (Hernando and Manso 1997). While it is not clear which signaling system(s) mediate these post-translational modifications of Hsp70, it has been suggested, as noted above concerning calcineurin, that this modification plays a role in the functional aspects of this protein (Joyeux et al. 1997). Post-translational modifications have also been reported to regulate the functional capabilities of other HSP. For instance, phosphorylation of myocardial Hsp27 is critical in the protection against actin fragmentation during I/R-injury (Loktionova and Kabakov 1998). Clearly, there are many signaling systems that coordinate the activation of HSP from the pre-transcriptional level to the production of functional protein and the specific roles of these systems in the exercise response of skeletal muscle have barely been addressed.

PHYSIOLOGICAL IMPORTANCE OF HSP IN SKELETAL MUSCLE

Ischemia-Reperfusion

While numerous cardio-protective roles of the HSP during I/R injury have been established (see elsewhere in this book), the protective nature of these proteins in skeletal muscle is less clear. For example, rats subjected to heat shock preconditioning (core temperature increase to 42°C for 20 min) 24 and 48 h prior to 4 h of gracilis muscle ischemia followed by 3 h of reperfusion did not exhibit enhanced muscle viability (measured by NBT staining) afterwards, even though Hsp70 was elevated (Lille et al. 1999). In contrast, rats subjected to a localized hindlimb heating protocol (42°C for 20 min) associated with a significant increase in Hsp70, showed enhanced gracilis and tibialis muscle viability 24 h after a 3 h ischemic bout (Lepore et al. 2000). To further emphasize the difficulties in interpreting these results, when the authors of the latter study examined the effect of Hsp70 overexpression in non-differentiated (cultured myoblasts) and differentiated (myocytes or myotubes)

muscle cells, only the myoblasts overexpressing Hsp70 were protected against thermal and oxidative stress type insult (Lepore et al. 2000). Differences in the methodologies of these studies highlight important considerations when observing the protective nature of HSP. For example, the heat shock model in the former study, i.e. whole body instead of isolated limb hyperthermia, may induce systemic signals that influence not only heat shock protein expression, but also other biological mechanisms that in turn may influence protection against a subsequent insult. Also, when observing skeletal muscle ischemia, the time of ischemia (4 versus 3 h) and reperfusion (3 versus 24 h) versus when the protection is observed is important, especially given the dependence of Hsp70 on ATP to release substrate (Palleros et al. 1991). Ischemia causes a time dependent decrease in intracellular high energy phosphate stores in skeletal muscle that is potentiated during early reperfusion (Walker 1991). As mentioned previously, during the latter stages of ischemia and during reperfusion, substrates bound to Hsp70 would not be released and hence would be unable to perform normal roles. Consequently, function during early periods may be compromised or show no improvement. Later on, however, as ATP returns to basal levels and the Hsp70 bound proteins are now released, the cell (and consequently tissue) will regain a more normal functional state. Additionally, the type of preconditioning appears to be important as ischemic preconditioning (Lepore and Morrison 2000) does not appear to confer the same ischemic injury resistance as heat shock (Lepore et al. 2000) although, once again, the endpoint being observed appears to be important (see below).

Fatigue

Following sustained rigorous muscle contraction or exercise, muscular force production is not fully recovered for some time. This detriment in force production is more pronounced when observed under low frequency stimulation and consequently referred to as low frequency fatigue (Edwards et al. 1977). While the exact reasons for this are not fully understood, it has been proposed that disruption of the excitation-contraction coupling mechanism due to increased oxygen and nitrogen radical formation as well as altered Ca^{2+} handling are important (Callahan et al. 2001; Davies et al. 1982; Essig and Nosek 1997). Consequently, any process that could scavenge increased mitochondrial respiration associated free radicals or protect vital excitation - contraction associated proteins and enzymes may lead to fatigue resistance and/or enhanced recovery following a fatiguing event (e.g. chronic stimulation or strenuous exercise). In fact, muscles from superoxide dismutase (the primary superoxide anion scavenger and antioxidant) overexpressing mice show fatigue resistance relative to wild type mice (Bruton et al. 2008). Moreover, isolated diaphragm bundles do not fatigue as quickly in the presence of various antioxidants (Reid et al. 1992). With respect to Ca^{2+} handling, as noted above, the HSP have been shown to be associated with the SR Ca^{2+} ATPase of fast twitch skeletal muscle (Tupling et al. 2004). Hence, the likelihood that the HSP could be involved

Table 2. Potential protective applications of elevated HSP in skeletal muscle

Role	Application	Potential method to increase HSP
Prevention/reduction of ischemia reperfusion injury	Diabetes, spinal cord injury, vascular disease, surgery	Exercise training/active lifestyle, heating, massage, pharmacology
Fatigue resistance	Aging, atheltic performance	Exercise training/active lifestyle
Protection against disuse/denervation atrophy	Bed rest, microgravity, spinal cord injury	Exercise training/active lifestyle, heating, massage or other modality, pharmacology
Improved muscular performance	Aging/atheltic performance	Exercise training/active lifestyle, pharmacology

in fatigue resistance is high. However, the available data in these areas is both limited and equivocal. For example, plantaris muscles from rats subjected to heat shock (41.5°C for 15 min) either 1 or 4 days prior to a fatiguing protocol at 40 Hz (5 min and 20 s), showed a detriment in contractile force during low frequency stimulation similar to non-heat shocked control rats at all time points measured after the fatiguing protocol (Thomas and Noble 1999). Moreover, diaphragm and soleus muscles from transgenic animals overexpressing both the inducible and constitutive isoforms of Hsp70 were not different than those muscles from control (i.e. wild-type) animals in their functional properties including size, maximum tetanic force, frequency to generate half maximum tetanic force and fatigueability to 5 min of intermittent high frequency/low frequency stimulation. However, EDL muscles from transgenic animals did appear to be more sensitive to caffeine as indicated by enhanced contractile force to a low frequency stimuli in the presence of this agent. Those authors attributed this to an association between Hsp70 and SR Ca^{2+} handling and in particular, function of the SR/Ca^{2+} ATPase (Nosek et al. 2000). Moreover, in an isolated preparation of developing mouse skeletal muscle myotubes, a prior hyperthermic bout (42°C, 30 min) is protective against DNP (2,4-dinitrophenol, a mitochondrial uncoupler) and A23187 (a calcium ionophore causing external calcium entry and Ca^{2+} related cell death) induced creatine kinase release and cell death (Maglara et al. 2003). As these events likely occur during fatiguing muscular contractions and this suggests that the HSP could be associated with fatigue resistance (Table 2).

Hypertrophy/Atrophy

During periods of disuse, skeletal muscle (primarily slow twitch postural muscle) rapidly undergoes remodeling including decreases in muscle weight, fibre size,

myonuclear number, and overall transformations from slow to fast muscle phenotypes (for review see Appell 1990; Booth and Criswell 1997). However, muscle atrophy is not necessarily a dire occurrence, since it can also be viewed as an adaptive mechanism in which the body saves energy by converting high maintenance muscles to less costly sizes and types, but the loss of skeletal muscle size and consequently function after as little as 3 days can be detrimental to those undergoing rehabilitation after disuse, during the treatment of specific neuromuscular disorders or in microgravity environments. HSP have been implicated in both phases (i.e. hypertrophy and atrophy) of muscle remodeling (Agarraberes et al. 1997; Beckmann et al. 1990). In general, unloading is associated with a reduction in HSP while reloading or overloading results in an elevation of HSP. For example, soleus muscle Hsp60 and Hsp70 are reduced after 2 weeks of hindlimb unweighting (Oishi et al. 2008), Hsp27 phosphorylation is significantly lower in 14 day unloaded or deafferentated soleus muscle (Kawano et al. 2007), and soleus muscle Hsp47 mRNA is reduced after as little as 1 day of hindlimb unloading (Oguro et al. 2006). Alternatively, overloaded plantaris muscle hypertrophy is associated with a concomitant increase in Hsp70 (Locke et al. 1994) although it has also been shown that muscle mass may increase in the absence of changes in Hsp70 (O'Neill et al. 2006; Oishi et al. 2004). Muscle atrophy generally occurs as a result of decreased rates of protein synthesis, increased rates of protein degradation (Thomason et al. 1989) and some combination of controlled myonuclear (Allen et al. 1999; Borisov and Carlson 2000) and cell death (Allen et al. 1997) at later stages. There is evidence that elevated Hsp70 protects cells against apoptosis (Volloch and Rits 1999). Consequently, it is likely that Hsp70 would be important in the preservation of muscle size and function during disuse. In fact, elevated levels of Hsp70 induced by a brief period of heat stress (i.e. 15 min at 42°C), significantly prevent soleus muscle atrophy after 8 days of hindlimb unweighting (Naito et al. 2000) and heat treatment associated with an increase in both Hsp25 and Hsp70 attenuates soleus immobilization atrophy (Selsby and Dodd 2005). Interestingly, nature seems to have already exploited this relationship. In the hibernating bat, the basal expression of Hsp70 is almost twofold greater during hibernation and early arousal (after hibernation) than in normally active periods. Though dormant for approximately 3 months, these bats show no muscle atrophy, which suggests that the increase in Hsp70 is protective (Lee et al. 2008). The changes in skeletal muscle HSP with disuse or loading, aging, training or injury are correlational at best. Whether the HSP are early signals necessary to invoke fibre type changes following changes in muscle type or simply act as stress markers remains to be determined. Moreover, as evidenced by the equivocal findings above, whether an increase in skeletal muscle HSP is truly beneficial has not been conclusively answered, although some benefit is intuitively likely. For example, exercise training with a concomitant increase in quadriceps Hsp70 is not sufficient to attenuate the aging associated decreases in muscle function (Kayani et al. 2008), however, old Hsp70 overexpressing transgenic rats show skeletal muscle functional properties similar to adult wild-type animals, suggesting a role in muscle preservation (Broome et al. 2006). Additionally, the application of HSP overexpression is limited by the physiological methods used to

induce these proteins. Physical activity is a potent inducer of skeletal muscle HSP, but not always feasible (e.g. long term immobilization) and, as mentioned above, there is a relatively quick return to basal levels of HSP (days) upon activity cessation.

Alternate HSP Therapies in Muscle

A stark generalization about HSP is that, at the cellular level, they are pro-survival. In fact, the association of these proteins with cell survival is such that overexpression of HSP has been associated with various forms of cancers (Daugaard et al. 2005; Jaattela 1999; Lattouf et al. 2006). Whether this relationship is causal or correlational remains to be determined, but suggests a confounding factor in the development of pharmacological agents (an active area of HSP investigation) that can induce HSP for protection. Nonetheless, a non-pathological increase in HSP has not only been associated with cellular survival, but also whole organ survival after transplant including the kidney (Guan et al. 2008; Redaelli et al. 2001, 2002b) and liver (Matsumoto et al. 2001; Redaelli et al. 2002a). Hence it is not unreasonable that muscle tissue or cell survival during surgery would also benefit from an increase in HSP and this appears to be the case. Heat preconditioning (core temperature elevated to 42°C and maintained for 5 min) in donor animals 24 h prior to allogenaic (taken from different animals of the same species) transplantation of rodent hindlimbs results in significantly less muscle edema and necrosis as measured by nitroblue tetrazolium staining (Baumeister et al. 2004). Moreover, ischemic preconditioning (2 × 30 min bouts of muscle ischemia induced by infeeding arterial and neural clamp) of the latissiums dorsi muscle immediately or 24 h prior to whole muscle flap elevation (i.e. ischemia) resulted in significantly improved muscle survival up to 1 week later (Carroll et al. 1997). Although muscle HSP was not examined in the latter study, ischemic preconditioning is known inducer of this response. Interestingly, and in keeping with the idea of muscle as an organ comprising different tissues, it has also been found that preconditioning by local heating of the rodent right flank to 43°C (3 × 30 min with 30 min at room temperature in between) 24 h prior to surgery (flap elevation leading to ischemia induced injury) significantly attenuated reductions in capillary perfusion and significantly reduced tissue necrosis and apoptotic cell death (Harder et al. 2005). These results were concomitant with an increase in Hsp32 in the preconditioned group and abolished with an Hsp32 inhibitor, not only suggesting the importance of this HSP as opposed to others but also that the microvasculature was an important target of protection during ischemia (Harder et al. 2005). At the cellular level, it has recently been shown that heat shock pre-treatment of human skeletal muscle myoblasts can inhibit apoptosis in vitro, and when transplanted into murine tibialis anterior muscle, results in decreased active caspase-3 and greater incorporation of human fibres into the murine muscle, a novel finding that has important implications in the treatment of some neuromuscular diseases (Riederer et al. 2008). Moreover, skeletal muscle myoblasts that have previously been heat shocked (42°C for 60 min) show less apoptosis and greater incorporation and survival in the heart

than non-heat shocked myoblasts after intracoronary infusion (Suzuki et al. 2000). This not only suggests a novel use of the heat shock response in skeletal muscle myoblast transplantation to improve cardiac muscle function, but also suggests that methods of keeping skeletal muscle HSP elevated may be beneficial against injury, aging and other muscle wasting situations.

CONCLUSION

Skeletal muscle is a major integrated organ, which must adapt to diverse stimuli in its locomotory role. In this regard, exercise-mediated expression of HSP may represent an important adaptive mechanism. The HSP profile in muscle is determined by a complex interaction of signaling pathways influencing both transcription and translation, such that activation of the heat shock response, exhibits organ and muscle fiber type differences which affects both the degree and threshold of response. Exercise has the potential to affect all levels of the integrated musculoskeletal system including the motor nerves, vasculature, connective tissue and the contractile apparatus itself. Exercise induced effects may both protect the organ and allow it to adapt and remodel in the face of external stimuli. While great strides have been made in understanding the importance of exercise-mediated changes in HSP in skeletal muscle, more research is needed.

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CHAPTER 18

CIRCULATING HSP70 AS AN ENDOGENOUS CYTOPROTECTOR?

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Abstracts Although classically regarded as being an intracellular molecular chaperone, many studies have reported that the 70 kDa stress protein can be released from a number of different cell types in the absence of cell death. Furthermore, we and others have reported the presence of Hsp70 in the circulation of normal individuals, and levels of this protein in the peripheral circulation can be increased by a number of physical and psychological stressors. The question is: what is the physiological role of circulating Hsp70? Extracellular Hsp70 has been reported to have a number of neuroprotective and cytoprotective properties, and we have shown that high serum levels of Hsp70 protect individuals with hypertension from the development and progression of cardiovascular disease. It might therefore be that extracellular members of the 70 kDa stress protein family have multiple roles in the maintenance of physiological homeostasis which extend beyond their more commonly accepted properties as immunoregulatory molecules. Perhaps this ubiquitous family of stress proteins could also be considered as exogenous cytoprotectors.

Keywords: Cardiovascular disease; cytoprotection; Hsp70; peripheral circulation; release mechanisms

Abbreviations: Hsp70, seventy kilo-dalton heat shock protein; HUVECs, human umbilical vein endothelial cells; IL-6, interleukin-6; TLR, toll like receptor; TNF-α, tumour necrosis factor-α

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INTRODUCTION

Heat shock (stress) proteins are evolutionarily ancient and highly conserved families of molecules that are present in, and essential to all prokaryotic and eukaryotic cell types. Their name derives from the original observations that their expression was induced following exposure of cells to elevated temperatures (Ritossa, 1962; Tissières et al., 1974). However, it has subsequently been demonstrated that many of these molecules are constitutively expressed, and those that are inducible can be induced by a variety of physical and biochemical stressors such as ultraviolet and γ-radiation, bacterial and viral infection, certain chemicals and drugs, hypoxia and glucose deprivation (Santoro, 2000). During steady state conditions, stress proteins fulfil a variety of functions including the intracellular assembly, folding and translocation of oligomeric proteins (Hightower, 1991). Under conditions of cellular stress they act as cytoprotective agents by binding mis-folded proteins and either protecting them from denaturation, or directing them towards denaturation (Hartl, 1996).

Stress Protein Release and Circulating Stress Proteins

Although for many years the perception has been that stress proteins are intracellular molecules that are only released from non-viable (necrotic) cells, it is now known that these molecules can be released from a variety of viable (non-necrotic) mammalian cells, including endothelial cells (Hightower and Guidon, 1989; Bassan et al., 1998; Liao et al., 2000). Furthermore, a number of laboratories have reported Hsp60 and Hsp70 to be present in the peripheral circulation of normal individuals (Pockley et al., 1998, 1999, 2000; Xu et al., 2000; Rea et al., 2001; Lewthwaite et al., 2002; Pockley et al., 2002; Njemini et al., 2003). Acute infection also increases serum levels of Hsp70, with Hsp70 levels correlating with levels of the inflammatory markers IL-6 and tumour necrosis factor-α (TNF-α) and, counter-intuitively, with levels of the anti-inflammatory cytokine IL-10 (Njemini et al., 2003). Exercise also induces the release of Hsp70 into the peripheral circulation of normal individuals (Walsh et al., 2001; Febbraio et al., 2002; Lancaster and Febbraio, 2005b), a response which is duration- and intensity-dependent (Fehrenbach et al., 2005). Physical (tail) shock (Johnson et al., 2005) and psychological stress induced by exposing male rats to a cat without physical contact has been shown to increase serum levels of Hsp70, and this effect occurs concurrently with an induction of intracellular expression of Hsp70 in the hypothalamus and dorsal vagal complex (Fleshner et al., 2004). This response appears to be mediated by adrenal hormones, as the induction on intracellular expression and circulating levels of Hsp70 elicited by cat exposure does not occur, or is attenuated, in adrenalectomised animals (Fleshner et al., 2004). The impact that circulating stress proteins have on pathophysiological processes, if any, is currently unclear.

A curious and as yet unexplained observation has been that individuals broadly segregate into those that exhibit low levels and those that exhibit high (in some cases

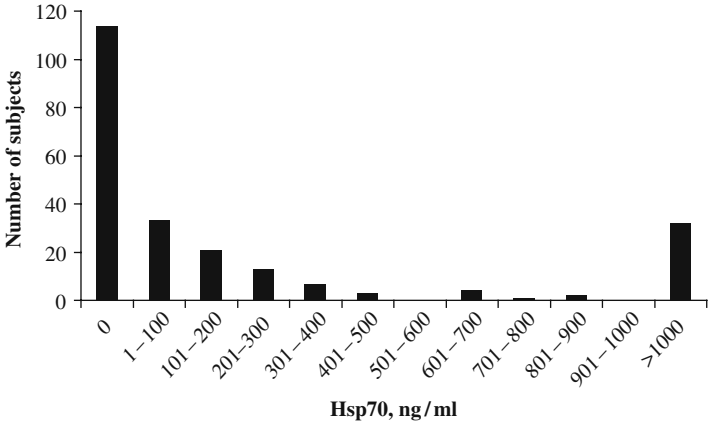

Figure 1. Distribution of serum Hsp70 levels in normal individuals ($n = 75$).

very high) levels of Hsp70 in their circulation (Figure 1). The mechanism underlying the presence of these distinct phenotypes have yet to be determined and it remains unknown whether they result from a differential production and release of these proteins or a differential rate of clearance from the circulation.

A number of factors appear to influence circulating levels of Hsp70 in overtly healthy individuals, one of which is age, as Hsp70 levels in peripheral blood lymphocytes and serum levels of Hsp70 are lower in the elderly and decline with aging (Rea et al., 2001; Jin et al., 2004; Terry et al., 2004). This observation is in keeping with the reduced capacity of cells and organisms to generate stress responses with aging (Faassen et al., 1989; Liu et al., 1989; Fargnoli et al., 1990; Luce and Cristofalo, 1992; Effros et al., 1994; Nitta et al., 1994; Heydari et al., 1995; Pahlavani et al., 1995; Richardson and Holbrook, 1996; Bernstein et al., 2000; Gray et al., 2000; Hall et al., 2000). Although the biological and physiological relevance of declining levels of circulating stress proteins with increasing age are unclear, an attenuated stress protein response might contribute to the increased susceptibility to environmental challenges and the more prevalent morbidity and mortality which is seen in aged individuals (Richardson and Holbrook, 1996; Shelton et al., 1999).

Release of Stress Proteins

To date, stress proteins such as Hsp70 have largely been studied in their context as intracellular chaperones, and their release from cells has typically been believed to occur only after lysis. However, it is now clear that such proteins can be released in the absence of overt cell necrosis, and these findings challenge many of the pre-conceived ideas surrounding the function and physiological role of extracellular stress proteins. A wider acceptance that stress proteins can be released from

cells in the absence of cell death requires a better elucidation of the pathways via which release/secretion is achieved. One of the difficulties with accepting the concept that stress protein release is a normal physiological event is that stress proteins do not express the typical N-terminal signal peptide sequences that are typically required for secretion. However, "non-classical" secretion of proteins that lack such sequences has been observed (Chimini and Rubartelli, 2005) and a lack of information on potential release mechanism(s) should not necessarily foster scepticism that such events occur. Much has yet to be understood about protein secretion mechanisms and it should be noted that the secretion pathways by which key mediators like IL-1 are released from eukaryotic cells are still not fully elucidated.

A number of laboratories have now provided insight into the potential mechanisms underlying the release of stress proteins such as Hsp70. Work from the Multhoff laboratory has demonstrated that detergent-soluble vesicles actively released by tumours contain high amounts of Hsp70/Bag-4 and Hsp70/Hsp40 (Gastpar et al., 2005). The biochemical and biophysical properties of these vesicles indicate that they are exosomes (Gastpar et al., 2005). Exosomal export of Hsp70 from tumour cells has also been reported by Asea's group (Bausero et al., 2005), and Hsp70-containing exosomes have been reported to be released by peripheral blood mononuclear cells (Lancaster and Febbraio, 2005a). It has also been proposed that cholesterol-rich microdomains lipid rafts, which are specialised membrane domains enriched in sphingolipids, cholesterol and proteins ("lipid rafts") are involved in the localisation of Hsp70 to the cell surface and its secretion into the extracellular environment (Broquet et al., 2003).

The release of stress proteins might also involve their entry into secretary lysosomal endosomes and their cell surface and release of the contents of the endolysosome into the extracellular space. It is certainly the case that febrile conditions induce the entry of Hsp70 into endolysosomes and their secretion in an extracellular ATP-dependent manner. The release of Hsp70 from tumour cells and macrophages has been shown to be mediated via such a route (Mambula and Calderwood, 2006). Phospholipase C inhibition might also be one of the mechanisms via which stress proteins are released, as a phospholipase C activity inhibitor (U731222) induces Hsp70 release from the A431 human carcinoma cell line (Evdonin et al., 2004). This is prevented by inhibiting vesicular transport with brefeldin A (Evdonin et al., 2006). But what might be the physiological basis for the release of stress proteins, and of what significance is their presence in the peripheral circulation? Although this question has yet to be definitivelt answered, some insight has arisen from studies in which the relationship between circulating Hsp70 and cardiovascular disease has been explored.

Circulating Stress Proteins and Cardiovascular Disease

Although circulating Hsp60 levels are not associated with cardiovascular risk factors such as body mass index, blood pressure and smoking status (Pockley et al., 2000),

Table 1. Relationships between circulating Hsp60 and Hsp70 levels and cardiovascular disease indices/risk factors[1]

Association with	Hsp60	Hsp70
Borderline hypertension	$P < 0.001$ (higher)	X
Diastolic blood pressure	$r = 0.34$; $P<0.001$	X
24 h systolic blood pressure	$r = 0.179$; $P=0.037$	X
Carotid intima-media thickness	$P < 0.009$	X
Very low density lipoprotein	$P < 0.017$	X
Triglyceride	$P < 0.05$	X

[1]Data are collated from Pockley et al. (2000), (2002) and Pockley et al. (2003).

levels are elevated in a subpopulation of patients with acute coronary syndromes (Zal et al., 2004). Levels are also associated with early atherosclerosis (on the basis of carotid intima-media thicknesses) (Pockley et al., 2000; Xu et al., 2000) and with serum concentrations of the pro-inflammatory cytokine TNF-α and other markers of inflammation in overtly healthy individuals (Lewthwaite et al., 2002). Circulating Hsp60 is higher in individuals exhibiting an unfavourable lipid profile (low high-density lipoprotein cholesterol and high total/high-density lipoprotein cholesterol ratio) (Lewthwaite et al., 2002) and levels are associated with very low-density lipoprotein and triglyceride concentrations (Pockley et al., 2000). In contrast, Hsp70 levels are not associated with very low-density lipoprotein and triglyceride concentrations (Pockley et al., 2000), and there is no relationship between Hsp70 levels and intima-media thicknesses in normal subjects, subjects with established hypertension, or subjects with borderline hypertension (Pockley et al., 2000, 2002) (Table 1). It would therefore appear that factors influencing circulating Hsp60 and Hsp70 levels are differentially regulated, despite the fact that overall, levels of Hsp60 and Hsp70 in the peripheral circulation correlate with each other (correlation coefficient 0.659, $P < 0.001$, $n = 366$).

An interesting and potentially important observation relating to the physiological role of circulating Hsp70 in cardiovascular disease has been made in a study of 218 subjects with established hypertension which has shown that increases in carotid intima-media thicknesses (a measure of atherosclerosis) over a 4-year follow-up period are significantly less prevalent (odds ratio 0.42; $p < 0.008$) in individuals having high serum Hsp70 levels (75th percentile, >300 ng/ml) at baseline (Pockley et al., 2003). The relationship between Hsp70 levels and changes in intima-media thickness was independent of age, smoking habits and blood lipids. Another study of 421 individuals evaluated for coronary artery disease has found that serum Hsp70 levels were significantly *higher* in disease-free patients (Zhu et al., 2003). Furthermore, healthy endarteries secrete more Hsp70 than carotid atherosclerotic plaques, and low plasma levels of Hsp70 are found in patients with atherosclerosis (Martin-Ventura et al., 2007). The source of circulating Hsp70, the mechanism(s) leading to its release and the association between circulating Hsp70 and atherosclerosis remain unknown.

The Cytoprotective Effects of Extracellular Stress Proteins

One of the earliest reports that stress proteins can be released from cells came from the Tytell laboratory which first demonstrated that glia-axon transfer proteins, which include Hsp70, Hsc70 and Hsp100, are transferred from adjacent glial cells to the squid giant axon (Tytell et al., 1986). The findings that extracellular Hsp70 can inhibit motor and sensory neuron degeneration (Tidwell et al., 2004; Robinson et al., 2005; Tytell, 2005) and that they enhance the tolerance of neuronal cells to stress (Guzhova et al., 2001) highlight its neuroprotective properties and suggest that the release of such proteins might be an altruistic response on the part of one cell for the protection of adjacent cells (Hightower and Guidon, 1989).

The cytoprotective effects of extracellular Hsp70 extend beyond neuronal protection, as it has also been shown to protect heat-stressed cynomolgus macaque aortic cells (Johnson et al., 1990) and serum-deprived rabbit arterial smooth muscle cells (Johnson and Tytell, 1993) from cytotoxic damage. Interestingly, the protective effects for serum-deprived rabbit arterial smooth muscle cells required cell association, but not internalisation. This observation implicates the involvement of receptor-ligand interactions and down-stream intracellular signalling events. Although the putative cell surface receptors involved in the cytoprotective properties of extracellular Hsp70 have not been identified, Hsp70 can bind to vascular cells via a number of potential receptors. Human Hsp70 has been shown to bind to human umbilical vein endothelial cells (HUVECs) (Thériault et al., 2005), although the receptors involved and the biological consequences have yet to be fully characterised. The Calderwood laboratory has reported significant binding of Hsp70 to the C-type lectin receptor LOX-1, but this did not explain the total binding capacity of these cells. Hsp70 binding to the "more established" receptors CD91, CD40, CD14, TLR2 or TLR4 was not apparent (Thériault et al., 2005). We have confirmed that Hsp70 binds to HUVECs, and also that it is rapidly internalised and localised (Pockley et al., 2009). We have also shown that Hsp70 binds to human dermal microvascular endothelial cells (HuDMECs), and that a larger proportion of HuDMECs bind Hsp70 (Pockley et al., 2009). It therefore appears that different endothelial cell populations express Hsp70 receptor(s) that are distinct to those which have been identified on antigen presenting cells of the immune system. It is possible that the physiological significance and functional consequences of Hsp70-endothelial cell interactions are dependent on the vascular compartment in which such interactions take place.

Some insight into the mechanism underlying the cytoprotective properties of Hsp70 has arisen from work demonstrating that exogenous Hsp70 increases intracellular Hsp70 levels and that this delays the decline in the viability of stressed arterial cells (Berberian et al., 1990). The capacity of extracellular Hsp70 to increase intracellular levels of Hsp70 might explain its cytoprotective effect, as Hsp70 prevents caspase-3 and SAPK/JNK activation in heat shock- or ceramide-induced apoptosis (Mosser et al., 1997; Ahn et al., 1999) and the cytoprotective effects of *intracellular* Hsp70 are established (Jäättelä et al., 1992; Simon et al., 1995; Samali and Cotter 1996; Lasunskaia et al., 1997; Mosser et al., 1997).

CONCLUSION

The discoveries that stress proteins such as Hsp70 can be released from cells by physiologically-relevant pathways, that these proteins are present in the circulation of normal individuals and that levels can be increased by exposure to stressors, infection and pathological disease poses questions concerning the physiological and functional relevance of circulating Hsp70. Experimental data indicate that members of the 70 kDa family of stress proteins can have a number of cytoprotective effects, and it might be that in addition to its well-documented properties as an immunoregulatory molecule, extracellular Hsp70 should also be considered as a physiologically-relevant, endogenous cytoprotector.

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CHAPTER 19

72 kDa EXTRACELLULAR HEAT SHOCK PROTEIN (eHsp72), NOREPINEPHRINE (NE), AND THE INNATE IMMUNE RESPONSE FOLLOWING MODERATE EXERCISE

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Abstract: It is now well known that both norepinephrine (NE) and 72 kDa extracellular heat shock protein (eHsp72) are released during stress, and that they can activate the immune system, mainly the innate immune response, even before a pathogen challenge. This is one reason why they have been postulated as "stress messengers or mediators" or "danger signals" for the immune system during stress. Exercise constitutes a stress because it alters the organism's homeostasis. Indeed, most of the exercise-induced changes in the immune system (including moderate *exercise*) are mediated by stress hormones and proteins, including NE and eHsp72. In this chapter, we present the latest studies performed in our laboratory about the role of NE and eHsp72 in the moderate-exercise-induced stimulation of neutrophil function, reviewing the main literature on the interaction between NE and Hsp72 not only in stimulating the innate immune response but also in the role of NE as a triggering signal in the stress-induced systemic release of eHsp72, particularly following moderate exercise. We also discuss the immunophysiological relevance of these interactions, as well as the optimal level of exercise that improves, but not impairs, the immune function by stimulating innate and/or inflammatory response mechanisms

Keywords: Catecholamines; Hsp70; neutrophils; immunity; stress; health

Abbreviations: ACTH, adrenocorticotropic hormone; APC, antigen presenting cells; PBMC, peripheral blood mononuclear cells; eHsp72, seventy two kilo Dalton extracellular heat shock protein; ERK, extracellular signal-regulated kinases; fMLP, formyl methionyl-leucyl-phenylalanine peptide; IL-6, interleukin-6; LPS, lipopolysaccharide; NE, norepinephrine; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; NK, natural killer cells; PI3K, Phosphoinositide 3-kinases;

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PRR's, pattern recognition receptors ; ROS, reactive oxygen species ; SNS, sympathetic nervous system ; SP, stress proteins ; TLR 2, toll like receptor 2

INTRODUCTION

The immune system is a system for self-recognition and maintaining homeostasis. It is an extremely complex network that extends throughout the body, and it can recognize and defend the organism against a theoretical infinity of challenges. In the innate immune mechanisms, macrophages and neutrophils participate, along with natural killer (NK) cells, complement, and defensins, to constitute the first line of defence. All its constituents need a basic capacity to distinguish between self and foreign, and danger or no danger. By picking up, processing, and presenting antigens, macrophages, together with antigen presenting cells (APC) in general, form the critical link to the specific or adaptive branch of the immune system that mainly consists of the various subpopulations of lymphocytes and their products. A few years ago it was thought that the immune system was a self-regulating physiological system. Nonetheless, today it is accepted as one of the three major regulatory systems together with the nervous and endocrine systems, communicating via neurotransmitters, neurohormones, hormones, and cytokines. Also, its functioning should be understood both in normal homeostatic conditions and when homeostasis is altered, as in sickness or in situations of stress.

EXERCISE, STRESS, IMMUNE SYSTEM, AND HEALTH

Exercise is a form of stress. In our laboratory, for more than two decades we have been studying the impact of physical activity on the immune system through "stress mediators or messengers" that are released when the activity is being performed. This is essential in order to recognize the beneficial (or harmful) effects of physical exercise on health. Indeed, the relationship between physical exercise, stress, and immunity is a good model of a neuroimmunoendocrine interaction. Thus, the effects of exercise on the immune system are mainly mediated by alterations in the sympathetic nervous system (SNS) and/or the hypothalamus-hypophyseal axis. In this sense, in many cases the results and conclusions drawn from immunophysiological studies of exercise, stress, and immunity seem paradoxical. Is exercise good or bad for health? Is stress good or bad for health? And is the release of stress hormones and proteins good or bad for health? When one accepts that physical exercise, even that of moderate intensity, is a stress situation, and that it is not necessarily either good or bad for the organism but will simply cause it to produce an appropriate response (adaptation) to the homeostatic changes naturally caused by the exercise, the mist begins to clear from the concepts.

Traditionally, exercising has been held to improve the health, and people who perform some type of sport regularly have been associated with having less susceptibility to infection and other pathologies compared with sedentary people, especially

if the sport performed is of low intensity. Although regular moderate exercise is very likely associated with decreased susceptibility to infection, recent years have seen a perhaps excessive generalization of the idea that, while moderate exercise is beneficial, intense exercise is harmful for the immune system. The latest studies, however, have revealed than this general finding cannot be extended to the innate immune response, and particularly to phagocytes. Some stages of the phagocytic process, chemotaxis and phagocytosis in particular, are stimulated by both moderate and intense exercise. In 1992 (Ortega et al., 1992), we postulated that the stimulation of macrophages during strenuous physical activity might counterbalance the decreased lymphoid activity, and that this may be regarded as an adaptation of the phagocytic cells to exercise-stress situations, in which there participate stress hormones. Thus, we suggested that phagocytes, and the innate defences in general, play a major role in the defence against infection during exercise-induced stress, probably preventing the entry and maintenance of microorganisms in situations where the specific response seems to be depressed (Ortega, 1994). However, while inflammation is necessary in host defence, uncontrolled inflammatory reactions are responsible for the initiation and progression of autoimmune and inflammatory diseases, especially in women, who are more susceptible to these kinds of pathologies (Wilder and Elenkov, 2001) probably because they have a "higher basal inflammatory status" in which there seem to be involved stress hormones and proteins such as norepinephrine (NE) and 72 kDa extracellular heat shock proteins (eHsp72) (Giraldo et al., 2008). The balance between pro- and anti-inflammatory cytokines is also critical in these pathologies (Elenkov and Chrousos, 2002).

The effects of exercise on resistance to infection also depend on when exercise is introduced in relation to the infection. It has been reported that exhaustive exercise training prior to an influenza infection increases survival time, but exhaustive exercise during or after infection decreased survival (Ilback et al., 1984, MacKinnon, 1992). We have suggested that the behaviour of the phagocytes may explain this, at least in part: the stimulation of the phagocytic activity could prevent infection during exercise, but if the organism is already infected, intense exercise could increase the infection due to a deficiency in the specific immune response (Ortega, 2003). Studies on animal-tumour models suggest the same behaviour with respect to the effects of exercise. It has been reported that exercise training before, but not after tumour development, reduces metastasis in rodents (MacNeil and Hoffman-Goetz, 1993). In addition, there is still controversy about the protective value of voluntary moderate (Cohen et al., 1993) versus forced intense (Thompson, 1994) exercise on breast cancer, above all when it is studied during or after the post-initiation stage of mammary carcinogenesis. The stress generated by forced activity is also an especially important consideration in interpreting the impact of exercise on tumour growth. Studies in our laboratory have clearly shown that exercise-induced stress enhances mammary carcinogenesis in experimentally-induced tumours in rats, with the involvement of epinephrine (E) and prolactin (Sáez et al., 2007), confirming the general idea that exercise-induced stress can be detrimental in unhealthy individuals. Nevertheless, although moderate exercise has frequently been recommended, human

epidemiological studies are inconclusive regarding the protective effect of physical activity on breast cancer in women (Hoffman-Goetz and Husted, 1994; Thompson, 1994).

We have said that in most cases exercise and the stress that is inherent to it are indistinguishable, and that the effects exercise induces in the immune response are mediated by stress hormones and factors (Khansari et al., 1990; Caren, 1991). Indeed, the interactions between the immune system and physical exercise are today regarded as a model of response to stress (Hoffman-Goetz and Pedersen, 1994), including the cellular non-specific immune response carried out by the phagocytes (Ortega, 1994, 2003). When we accept exercise as a model of stress, it becomes clear that no general statement that stress induces immunosuppression can be maintained for all levels of the immune system. Depending on concentrations and time of exposure, although stress hormones (mainly glucocorticoids and catecholamines) can impair some specific immune functions, they may also significantly stimulate different aspects of the innate immune and/or inflammatory response (Ortega, 2003). Recently, several laboratories have reported that exercise-induced stress also results in the release of the 72 kDa heat shock protein (Hsp72), which also has marked effects on immunity. This has given rise to a new wave of functional interpretations of the activation processes of the immune system by factors released in situations of stress ("stress mediators", "messengers of stress", and/or "danger signals"), even in the absence of any prior antigenic stimulus. While traditionally only intense exercise has been regarded as stressful, there is growing acceptance that moderate exercise also involves a situation of stress since it can also substantially alter homeostasis. Recent studies conducted in our laboratory have shown how both NE and eHsp72 also participate in modulating the immune response during exercise of moderate intensity – exercise which is more suitable for the quality of life and well-being of the healthy population and for complementary therapies in various pathologies.

In the following, we shall review from an immunophysiological point of view of the most important investigations relative to the role played by NE and eHsp72, both separately and together, in the modulation of the immune function, primarily in the innate and/or inflammatory response, hypothesizing about the consequences of the performance of moderate exercise for the health of those who practise it.

ROLE OF NOREPINEPHRINE (NE) IN THE NEUROIMMUNOMODULATION DURING MODERATE EXERCISE

Almost all the mechanisms involved in an immune response can be affected by noradrenergic neurotransmitters (Elenkov et al., 2000; Sanders, 2006), especially during stress. Exercise constitutes a stress, and the profile of release of stress hormones, including catecholamines, differs according to the intensity and duration of the exercise as well as of the previous training (or physiological adaptation). Although both E and NE may mediate the immune response during intense exercise, NE appears to be responsible during acute bouts of moderate-intensity exercise

(Brenner et al., 1998; Ortega et al., 2007). Indeed, during single bouts of moderate exercise conducted in our laboratory, we have only seen an increase in the blood concentration of NE (Ortega et al., 2005a, b), but no significant changes in the concentration of E. NE can inhibit or stimulate an immune response depending on the concentration and type of adrenergic receptor involved. Innate immune cells express both alpha- and beta-adrenergic receptors. However, T and B lymphocytes express adrenergic receptors of the β2 subtype exclusively, except for Th2 cells that lack expression of any subtype. By binding to adrenergic receptors, NE is able to induce changes in the level of cellular activity, which also regulates changes of gene expression for cytokines and antibodies (Nance and Sanders, 2007). In a recent review, Besedovsky and Del Rey (2007) indicate that the effect of NE also depends on the type of stimulus that triggers the immune response, the subset of cells affected, and, most importantly, at which step of the response lymphoid and/or accessory cells are exposed to neurotransmitters. Among the processes directly or indirectly affected by sympathetic neurotransmitters are phagocytosis by macrophages and neutrophils, antigen presentation, the expression of co-stimulatory and adhesion molecules, lymphoid cell activation, cytokine production, and the generation of cytotoxic cells (Besedovsky and Del Rey, 2007; Ortega et al., 2007). Studies in our laboratory have also demonstrated that the modulation of lymphocytes (García et al., 2003a) and macrophages (Sáez et al., 2002; Ortega et al., 2000a, b; García et al., 2003b) by NE not only depends on the concentration of this neurotransmitter, but also on the age and in the longer term possibly on the NE catabolic products too, such as 4-hydroxy-3-methoxyphenyl-glycol (HMPG).

Moderate exercise benefits the immune system (Pedersen and Hoffman-Goetz, 2000). It stimulates the function of lymphocytes (Fitzgerald 1988), natural killer cells (Pedersen et al., 1998), and phagocytic cells including macrophages and neutrophils (Ortega, 1994). Studies in our laboratory are coherent with NE mediating both the quantitative (number) and qualitative (function) stimulation of innate immune cells during short-term acute moderate exercise. Moderate exercise (45 min at 55% VO_2 max on an ergometric cycle) provokes in sedentary men changes in all leukocyte subpopulations, although the behaviour of monocytes ($CD14^+$) and granulocytes ($CD15^+$) was different from that of lymphocytes (Ortega et al., 2005a). Phagocytes increased the number from the beginning of exercise and reached the highest values immediately after finishing it, with no decrease during the recovery period, and this correlated with the plasma concentration of NE. Although the behaviour of lymphocytes and NK cells was consistent with the "open window" theory, the behaviour of neutrophils and monocytes (as previously had been reported for higher exercise intensities: Pedersen et al., 1998) may counteract this "open window" situation under the mediation of NE (Ortega et al., 2005a). During this model of exercise, sedentary individuals did not show significant changes in E and cortisol concentrations, which agrees with the idea that following moderate exercise the increase in leukocyte number is related mainly to the plasma NE concentration, but during more intense exercise E concentration takes on a greater importance, as also does cortisol with long-duration exercises (Brenner et al., 1998; Pedersen et al., 1998; Ortega

et al., 2005a). The participation of NE in the modulation of the functional capacity of neutrophils during single bouts of moderate exercise has been confirmed in studies with untrained men, showing that the increase in the concentration of systemic NE after the exercise stimulates the phagocytic capacity of neutrophils (Ortega et al., 2005b) and participates indirectly in stimulating their microbicidal capacity (Ortega et al., 2006), in both cases with the participation of both alpha- and beta-adrenergic receptors. Also, the NE not only participates in the capacity for phagocytosis and destruction of antigens by neutrophils, but can also participate in the moderate exercise induced stimulation of the mobility and capacity of neutrophils to approach foci of infection (Fig. 1). *Ex vivo* and in vitro studies show that the release of NE generated during a session of moderate exercise leads to the stimulation of neutrophil chemotaxis when confronted with known chemoattractants (such as formyl methionyl-leucyl-phenylalanine peptide, fMLP), a fact that is coherent with the finding that, after incubation of phagocytes with the "post-exercise" concentration of NE, their chemotactic capacity is significantly greater than seen with the basal concentration (Fig. 2a). This mediation of NE in the stimulation of neutrophil chemotaxis has also been found in non-active women, with the necessary participation of both alpha- and beta-adrenergic receptors (Ortega et al., 2007). The role of NE in regulating the migration of neutrophils to inflammatory foci is not only reflected in its being an agent activating chemotaxis, but also through its chemoattractant capacity (Fig. 2b): basal NE concentrations in young men and the concentrations released during moderate exercise present a chemoattractant capacity for neutrophils, at levels even higher than fMLP. Also, the chemoattractant capacity of the post-exercise concentration of NE is significantly greater than that presented by the baseline concentration, clearly suggesting that this neurohormone is a signal activating the movement of neutrophils during exercise, probably allowing these cells to reach inflammatory foci in situations of alert for the organism. These results confirm the idea that catecholamines (with more relevance for NE during moderate exercises) may physiologically stimulate the neutrophil functions, such as chemotaxis and phagocytosis, and strongly suggest that NE participates as a "stress mediator" in the moderate exercise induced stimulation of neutrophil function (Ortega et al., 2007). This also supports the hypothesis that sympathetic nerve activity is generally suppressive for immunocompetent cells in the blood stream, except for neutrophils (Nagatomi et al., 2000).

Nevertheless, during intense training performed by competitive sportspeople, both E and NE are involved. In highly trained cyclists at rest, we have observed a strong correlation between the greater percentage of monocytes and neutrophils with phagocytic capacity against *E. coli* (measured by flow cytometry) and the E concentration during the central months of a training season. However, we found a decline in the systemic concentration of NE in parallel with the greater percentage of monocytes and neutrophils with phagocytic capacity. One might hypothesize that a transitory decline in NE levels may eliminate an immunosuppressory effect and allow a greater number of active phagocytes, constituting an adaptation to training (Ortega et al., 2001). These results suggest that the effect of NE on phagocytes may not be the same

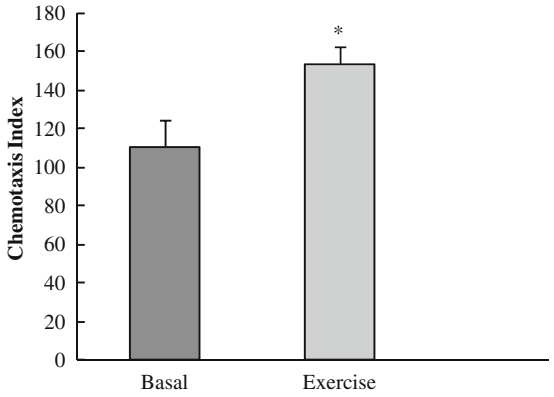

Figure 1. Influence on neutrophil chemotaxis of a single bout of moderate exercise (45 min cycling on an ergometric cycle at 55% VO_2 max) performed by sedentary young men. Blood samples were taken before (basal values) and immediately after exercise. Chemotaxis was evaluated on isolated neutrophils in a Boyden chamber using an fMLP gradient. The chemotaxis index represents the number of neutrophils counted at random (under 100× phase contrast microscopy) in 16 fields of the lower part of the filter (for details of the technique see Ortega et al., 2009). Each column represents the mean ± S.E.M. of 10 independent experiments performed in duplicate (one experiment for each volunteer; all experiments gave similar results) $^*P < 0.01$ vs. basal values (Student *t*-test)

during the adaptation to a training program or "chronic exercise" as in response to a single bout of exercise.

Results on macrophages have been more controversial. Both intense exercise (Forner et al., 1995; Ortega et al., 1997) and NE (García et al., 2003b) stimulate the phagocytic process of macrophages (the least specific function of this cell), but they may suppress macrophage antigen processing (Ceddia et al., 2000; Woods, 2000), a more specific aspect of macrophage function. It has also been reported that stress, including severe exercise, modulates the systemic and/or local pro-/anti-inflammatory balance (Elenkov and Chrousos, 2002). Although NE (through beta-adrenergic receptors) inhibits the production and secretion of TNF-α by splenic and lymph node macrophages in response to lipopolysaccharides (LPS) (Ignatowski et al., 1996), the results for IL-1β are less consistent (Meltzer et al., 2004). Moreover, both inhibitory and facilitatory effects of NE on IL-6 production have been reported (Nance and Sanders, 2007; Meltzer et al., 2004). Although there have been no studies in this respect during moderate exercise, the fact that during single bouts of moderate exercise the levels of NE also increase allows one to hypothesize that, during these sessions, NE may also modulate the balance of the production of inflammatory cytokines by monocytes/macrophages. Indeed, studies in our laboratory have shown a better balance of the systemic release of pro-/anti-inflammatory cytokines during the moderate intensity exercises than during a session of high intensity. For example, studies in non-active women have recently found that following a single bout of moderate cycling (45 min at 55% VO_2 max) the serum concentrations

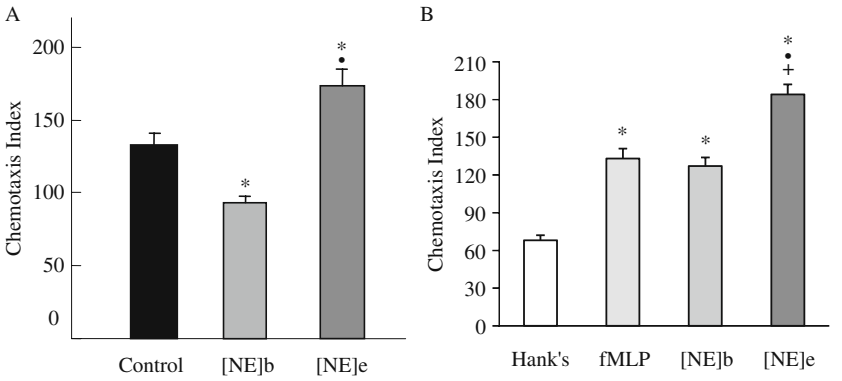

Figure 2. *Left part* (**a**) shows the in vitro effect on neutrophil chemotaxis of NE released into the blood following a single bout of moderate exercise (55% VO_2 max during 45 min on an ergometric cycle) performed by sedentary men. Neutrophils were incubated with NE at 0 (control), 0.39 ng/ml (basal concentration, [NE]b), or 0.87 ng/ml (post-exercise concentration, [NE]e) in the upper part of the Boyden chamber. In the lower part of the chamber, 10^{-5} MfMLP was added. *Right part* (**b**) shows the chemoattractant capacity of NE. In this study, fMLP (10^{-5} M; a chemoattractant peptide), the basal concentration of NE ([NE]b, 0.39 ng/ml), or the post-exercise concentration of NE ([NE]e, 0.87 ng/ml) were put into the lower compartment of the Boyden chambers. Control values were those obtained only with Hank's medium (with no chemoattractant property). The chemotaxis index is the number of neutrophils counted at random (under 100× phase-contrast microscopy) in 16 fields of the lower face of the filter (for more details of the methods of chemotaxis see Ortega et al., 2009). Each column represents the mean ± SEM of 10 independent experiments performed in duplicate (each experiment was performed on neutrophils isolated from 10 volunteers in their basal state; all experiments gave similar results). $^{*}p < 0.01$ vs. control values; $^{\bullet}p < 0.01$ vs. values obtained after incubation of neutrophils with [NE]b (ANOVA Scheffe *F*-test) in Fig. 2a. $^{*}P < 0.01$ vs control values (Hank's medium). $^{\bullet}P < 0.01$ vs. [NE]b values; $^{+}P < 0.01$ vs. fMLP values. (ANOVA-Scheffe *F*-test) in Fig. 2b

of the pro-inflammatory cytokine IL-1β, IL-6 and the anti-inflammatory cytokine IL-4 are increased. However, intense cycling (1 h at 70% VO_2 max) increased pro-inflammatory cytokines (IL-1β, IL-6, IL-12 and IFN-γ) but decreased the anti-inflammatory IL-4. This clearly suggests a better pro-/anti-inflammatory balance during moderate exercise, in parallel with an elevated plasma concentration of NE (but not E), while both catecholamine (E and NE) concentrations were augmented in the blood following intense exercise.[1] In young untrained men, a "good balance" between systemic pro- and anti-inflammatory cytokines has also been

[1] Data presented at the 2nd Iberoamerican Congreso of Neuroimmunomodulation (Madrid, Spain, 2007). Giraldo, E., Hinchado, M.D., García, J.J. and Ortega, E. (2006) Neuroimmunomodulation 13: 247–248 (Abstract); Giraldo, E., García, J.J., Hinchado, M.D., and Ortega, E. (2008) Exercise intensity-dependent changes in the inflammatory response in sedentary women: phagocytic process of neutrophils and pro-/anti-inflammatory cytokine balance. Role of neuroendocrine parameters. Neuroimmunomodulation (accepted for publication. DOI:10.1159/000212384).

Table 1. Changes in the serum concentration of pro- and anti-inflammatory cytokines following moderate exercise

Cytokines		Time after exercise (pg/ml)	
(expected values in health people)	Basal (pg/ml)	0 h	24 h
IFNγ (<10 pg/ml)	1.54 ± 0.54	2.16 ± 0.59	2.21± 0.64
TNF α (<10 pg/ml)	3.77 ± 0.85	3.21 ± 0.20	6.68 ± 0.67
IL-1β (0–15 pg/ml)	6.5 ± 0.2	9.1 ± 1.1*	9.2 ± 0.1*
IL-8 (0–47 pg/ml)	1.97 ± 0.69	0.73 ± 0.56	2.7 ± 0.62•
IL-12 (10–89 pg/ml)	97 ± 17	103 ± 20	152 ± 16*,•
IL-2 (0–1.16 U/ml)	0.92 ± 0.07 (U/ml)	0.92 ± 0.03 (U/ml)	–
IL-6 (<10 pg/ml)	2.26 ± 0.14	3.70 ± 0.37*	2.71 ± 0.13•
IL-4 (0–13 pg/ml)	all < 5 pg/ml 3.6 ± 0.49	all > 5 pg/ml 7.5 ± 0.96 *	–
IL-10 (0–112 pg/ml)	5.8 ± 0.9	4.5 ± 0.6	6.05 ± 0.84
IL-13 (0–87 pg/ml)	7.6 ± 3	7.0 ± 6.0	5.68 ± 0.2

The data show the variations in pro- and anti-inflammatory cytokines after a single bout of moderate exercise (45 min at 55% VO_2 max on an ergometric cycle) performed by non-active healthy young men compared with their basal status. Serum cytokines were measured by ELISA. Each value represents the mean ± SEM of 10 independent experiments performed in duplicate (one experiment for each volunteer; all showed similar behavior). The results show that moderate exercise increases the systemic concentration of the pro-inflammatory cytokines IL-1β (which remained elevated 24 h later), and IL-8 and IL-12 (only 24 h after exercise). This is counterbalanced with the elevated levels of IL-4, an anti-inflammatory cytokine, immediately after exercise. IL-6 also increased following moderate exercise and returned to the basal values 1 day later. $^*p < 0.01$ vs. basal values; $^\bullet p < 0.01$ vs. values immediately after exercise (ANOVA Scheffe *F*-test).

found in parallel with increased NE following a single bout of moderate exercise (Table 1).

Studies in phagocytes, then, do not always support the idea of a general immunosuppressive effect of catecholamines, and the general statement that catecholamines induce immunosuppression of all immune cells is no longer accepted as valid (Nagatomi et al., 2000; Ortega, 2003). This may be of particular importance in protecting the organism during exercise when minor injuries can permit bacteria to enter through disrupted skin and mucous membranes (Nagatomi et al., 2000, Ortega, 2003). However, although physiological concentrations of NE also stimulate neutrophil microbicide capacity (measured against *C. albicans*) through both α- and β-adrenoreceptors, it did not mediate directly the increased killing of *C. albicans* during moderate exercise, this effect being mediated by 72 kDa extracellular heat shock proteins (eHsp72). Thus, NE, and above all eHsp72, seems to be involved in the stimulation of the neutrophils' microbicide capacity during moderate exercise (Ortega

et al., 2006). Therefore, it is natural to speculate that during moderate exercise both NE and eHsp72 are also "danger signals" for the immune system, stimulating or facilitating the innate response. One may even speculate that, given the rapid release of NE, its systemic release constitutes a prior signal of stress and danger that subsequently will induce the systemic release of Hsp72. With this in mind, and before starting to assess the effects of eHsp72 on the innate immune response, we shall briefly discuss the important part that the heat shock proteins can play during situations of stress, and analyze the possible role of NE in their release.

EXTRACELLULAR HSP70 DURING EXERCISE-INDUCED STRESS

Exercise-Induced Release of Hsp72: Role of NE as Releasing Signals

The heat shock or stress proteins (Hsp or SP) were originally described as "molecular chaperones" with important roles in cellular transportation, assembly/degradation, and cell survival. Among them, of especial importance is the Hsp 70 family (e.g., Hsp72) in cell protection following exercise and exercise training (Locke and Noble, 1995). More recently, it has been demonstrated that exogenously added Hsp72 possesses potent chaperone and cytokine activity, a term that has been referred to as the "chaperokine" capacity of Hsp72 (Asea et al., 2000; Asea, 2005), suggesting an important role of eHsp72 during stress situations. It is now well known that blood concentrations of eHsp72 increase during stress, including exercise, facilitating the innate immune response. Walsh and co-workers (2001) were the first to demonstrate that Hsp72 is released into the blood during intense exercise (1 h at 70% of VO_2 max), and recent studies have shown that both intensity and duration of exercise influence the concentration of Hsp72 released into the circulation (Fehrenbach et al., 2005; reviewed by Whitham and Fortes, 2008). Thus, serum/plasma eHsp72 increase immediately after all types of exercises (endurance training, intense exercise at 70–80% VO_2 max, moderate exercise at 60% VO_2 max, or a highly intense competitive marathon run). Nevertheless, the increase in the eHsp72 blood concentration is even higher after long-lasting endurance exercise than after more intense but shorter exercise. It is also higher after single bouts of intense exercise than after single bouts of moderate exercise of the same duration (Fehrenbach et al., 2005). Although most of the studies showing exercise-induced increases in eHsp72 concentration have been performed at a high intensity and/or in trained people, recent studies in our laboratory have shown that single bouts of both moderate (45 min of cycling at 55% VO_2 max) and intense (1 h of cycling at 70% VO_2 max) exercise increase the blood concentration of eHsp72 in sedentary or non-active healthy men and women (Ortega et al., 2006, 2009). However, it was curious to observe that while, after 24 h of intense exercise performed by women, the blood eHsp72 concentration returned to the basal values (Ortega et al., 2009), following moderate exercise performed by men, the concentration remained high with respect to the basal values (Ortega et al., 2006). Therefore, the intensity and duration of exercise also seems to affect the time during

which the blood concentration of eHsp72 remains high, a time which seems to be highest after moderate intensity exercise.

However, the signal(s), the source(s), and the secretory pathways in the release of circulating eHsp72 have yet to be clarified. Two mechanisms have been proposed for secretory pathways: (a) passive release from an intracellular pool as a consequence of cell necrosis when tissue damage occurs (Gallucci et al., 1999), and (b) alternatively (or even additionally) active release in the absence of lysis (Hightower and Guidon, 1989) by a receptor-mediated exocytotic pathway (reviewed by Johnson and Fleshner, 2006; Asea, 2006; Whitham and Fortes, 2008). The more plausible of these possibilities is that eHsp72 must be actively released by exocytosis involving exosomes, for example from B cells (Clayton et al., 2005), tumour cells (Gastpar et al., 2005), and peripheral blood mononuclear cells (Lancaster and Febbraio, 2005), as well as intact surface membrane lipid rafts (Bausero et al., 2005) in the absence of cellular lysis or death. Thus, the passive release from necrotic cells is unlikely during exercise, above all during moderate exercise (reviewed by Johnson and Fleshner, 2006, Asea, 2005, 2006; Whitham and Fortes, 2008).

A wide variety of physiological signals induced by exercise are known to be capable of stimulating the induction of intracellular Hsp, including E and NE (Matz et al., 1996; Heneka et al., 2003; Maloyan and Horowitz, 2002), glycogen deprivation (Febbraio et al., 2002), oxidative stress (Marini et al., 1996), and heat or hyperthermia (Kregel, 2002; reviewed by Johnson and Fleshner, 2006 and Whitham and Fortes, 2008). Hyperthermia seems to be necessary not only for Hsp72 to be detected in cells (e.g., lymphocytes) during exercise (Chen et al., 1995), but it is also an essential factor for exercise-increased eHsp72 (Horowitz and Robinson, 2007; Ogura et al., 2008). It is also important for the exercise-induced systemic release of NE (Peake et al., 2008), suggesting a hypothetical influence of NE in the release of eHsp72 during an exercise-induced increase in body temperature. Indeed, it has been suggested that eHsp72 release could be mediated by signals associated with the hypothalamic-pituitary-adrenal or sympathoadrenal medullary axis, being part of a physiological stress response (Whitham and Fortes, 2008). Nevertheless, although ACTH (Blake et al., 1991), corticosterone (Sun et al., 2000), and catecholamines such as E (Matz et al., 1996) and NE (Udelsman et al., 1994) have been shown to be stress signals for the increase of intracellular Hsp72 in different tissues (reviewed by Whitham and Fortes, 2008), a stress signal role has only been demonstrated for the catecholamines in the extracellular release of Hsp72 (Fleshner et al., 2004). Johnson and co-workers (2005) in a very interesting series of experiments, demonstrated in rats that adrenergic receptors, mainly β_1-receptors, are involved in the stress-induced increase in eHsp72, hypothesizing than NE may stimulate a receptor-mediated exocytotic pathway of eHsp72 release during stressor exposure which results in sympathetic nervous system activation and the release of NE. Although elevated levels of both NE and E were found in the rats following tail shock stress exposure, those authors suggest that NE (rather than E) is the mediator of Hsp72 release, since it has a higher affinity for α_1-adrenergic receptors, and adrenalectomy had no effect on Hsp72 release after stress (Johnson et al., 2005; Fleshner

and Johnson, 2005). Nevertheless, the cell source and the mechanism by which NE mediates the release of Hsp72 need to be clarified.

Although moderate exercise was not classically considered to be a stress situation, today it is accepted that most of the effects of moderate exercise on the immune system are also mediated by "stress hormones" (reviewed by Ortega, 2003; Ortega et al., 2007). Long duration moderate cycling (180 min at 60% VO_2 max) has been reported to increase eHsp72 blood levels in humans, probably released from the brain (Lancaster et al., 2004). Blood concentrations of NE and eHsp72 also increase following short duration moderate cycling (45 min) performed by sedentary people (Ortega et al., 2006), confirming that a single bout of moderate exercise also constitutes a stressor in non-active humans. It has been reported that the increase in circulating eHsp72 is produced within 10–30 min of the onset of various whole-organism stressors (Fleshner and Johnson, 2005), a time that is longer than that necessary for the release of NE. Thus, it may be speculated that during exercise (including moderate exercise) the induction of a rapid release of NE into the circulation would constitute the first "danger signal" for the organism, and subsequently NE could be the signal for the release of eHsp72, probably involving circulating leukocytes in the absence of cell necrosis. In support of this hypothesis, it has been found that peripheral blood mononuclear cells (PBMCs) are continually releasing Hsp70 into the extracellular medium, and this release is not due to cell damage. The release is greater with raised temperatures (also suggesting that it occurs during exercise-induced increases in body temperature), with a contribution of 70% from B-lymphocytes and 30% from T-lymphocytes via a non-classical pathway which may involve lipid rafts (Hunter-Lavin et al., 2004). In contrast, data of Lancaster and Febbraio support the idea that exosomes (but not lipid rafts) contribute to Hsp70 release from PBMCs, even under basal conditions (Lancaster and Febbraio, 2005). There is no information available about the role of polymorphonuclear neutrophils as a cell source for eHsp72 release. It has been suggested that NE, acting upon α_1-adrenoreceptors, results in a calcium flux within the cell and the subsequent release of Hsp72 through exosomes, and then increased blood eHsp72 concentration (Fleshner and Johnson, 2005). Since the post-exercise NE concentration in blood does not induce any increase in intracellular Ca^{2+} concentrations in neutrophils (Ortega et al., 2005b), the hypothetical participation of neutrophils in the release of eHsp72 induced by NE seems unlikely, unless the mechanism is not via exosomes and/or a calcium-dependent pathway (perhaps mechanisms of necrosis or apoptosis). We are presently designing studies in this line.

Although the studies of Johnson and Fleshner (Johnson et al., 2005) were performed on rats, a role for catecholamines as stress-signals in the release of eHsp72 seems more than probable in humans also. Whitham and co-workers (Whitham et al., 2006) have reported a significant increase in eHsp72 in parallel with E (but not NE) in trained men stimulated with caffeine (a known stimulator of sympathetic activity) in response to prolonged intense exercise (two trials 90 min

cycling at 74% VO_2 max). However, during single bouts of moderate exercise performed by non-active men the role of NE is more prominent, since the circulating concentration of eHsp72 is increased in parallel with an increased NE (but not E) concentration (Ortega et al., 2006). It is possible that different patterns of sympathetic activation both across species and between different intensities and duration of exercise may be taking place in the interactions between catecholamines and Hsp72, as has been suggested for stress in general (Whitham and Fortes, 2008).

ROLE OF EHSP72 AS "STRESS MEDIATOR" OR "MESSENGER OF STRESS" IN THE STIMULATED INNATE RESPONSE DURING MODERATE EXERCISE

Hunter-Lavin and co-workers (2004) suggested that most serum Hsp70 comes from PBMCs. They posed the following question: Is the amount of Hsp72 released during stress significant in terms of cellular response to the protein? To answer this question and evaluate the immunophysiological role of eHsp72 in the neutrophil function during exercise, in our laboratory we designed *ex vivo* and in vitro experiments based on: (1) the direct effect of exercise on neutrophil function, (2) the determination of blood eHsp72 concentrations following exercise, and (3) in vitro incubation of neutrophils with the physiological concentration of eHsp72 measured in a basal situation versus the raised physiological levels following exercise. Several control studies were performed to test the specificity of the effects of Hsp72, and to establish that the effects were not due to possible endotoxin contamination in the commercial samples: (a) neutrophils were incubated only with the corresponding maximal LPS concentration contaminating commercial Hsp72, (b) polymyxin B, an inhibitor of LPS was added immediately before the Hsp72, and, as another control trial, (3) Hsp72 was maintained at 100°C for 90 min before stimulation of the neutrophils, which denatures the Hsp72 but not LPS.

Our first evidence of the role of eHsp72's as "stress mediator" in the activation of the innate immune response during a single bout of moderate exercise came from observing a sustained increase in neutrophil phagocytosis 24 h after the exercise when, however, the NE had returned to baseline values, suggesting that other neuroendocrine mechanisms (also independent of epinephrine and cortisol) had to be involved, at least during the recovery period (Ortega et al., 2005b). Subsequent studies (Ortega et al., 2006) showed that the stimulation of the neutrophils' microbicide capacity (against *C. albicans*) induced by the moderate exercise was also maintained for 24 h as was the raised blood concentration of eHsp72. This suggested that eHsp72 could play a more prominent role than NE in the stimulation of the microbicide capacity of neutrophils during a single bout of moderate exercise performed by untrained men. This role was confirmed in vitro, as the post-exercise eHsp72 concentration induced higher neutrophil microbicide capacity than the basal concentration.

We suggested that NE could serve as a signal that mediates the elevation of Hsp72 in the circulation during the moderate exercise performed by sedentary men, with both of them being involved in the microbicide capacity of neutrophils (Ortega et al., 2006). In addition, after the moderate exercise, the production of ROS (superoxide anion) by neutrophils also increased, contributing not only to the increased oxygen-dependent microbicide capacity of the neutrophils (Ortega et al., 2006), but perhaps also to inducing the expression of Hsp in this cell type, as has been reported for other immune cells such as lymphocytes (Marini et al., 1996). Further studies are clearly needed in this line.

Although microbicide capacity of phagocytic cells is probably the most important stage of the phagocytic process in order to destroy an antigen, it is essential that the phagocytes previously approach the focus of infection or inflammation to phagocytose the antigen. Recent studies in our laboratory have shown that the eHsp72's also participate as well as NE (Ortega et al., 2005b) in stimulating the phagocytosis of *Candida albicans* induced by a single bout of moderate exercise in sedentary men, since the post-exercise blood concentration of eHsp72 was sufficient to stimulate in vitro the phagocytic capacity of neutrophils with respect to the basal concentration (Fig. 3). The stimulation of phagocytosis by the post-exercise physiological concentrations of eHsp72 (found after single bouts of moderate or intense exercise) needs the participation of TLR-2 and intracellular signaling via ERK, PI3K, and

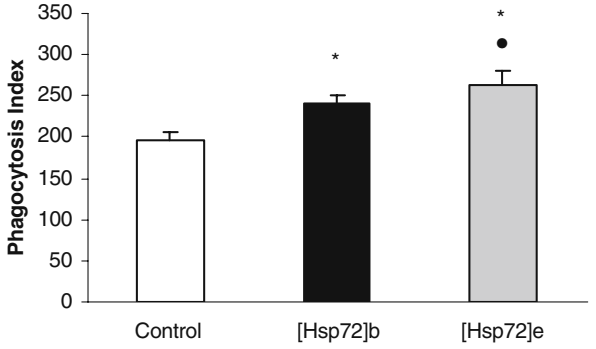

Figure 3. In vitro effect on neutrophil phagocytosis of Hsp72 released into the blood following a single bout of moderate exercise (55% VO_2 max for 45 min on an ergometric cycle) performed by sedentary men. The phagocytosis was evaluated against *C. albicans*. Both isolated neutrophils and *C. albicans* were adjusted to 10^6 cells/ml and incubated for 1 h in the presence of Hsp72 at 0 ng/ml (control), 5.12 ng/ml ([Hsp72]b, basal concentration) or 7.66 ng/ml ([eHsp72]e, post-exercise concentration). Each column represents the mean ± SEM of 10 independent experiments performed in duplicate (each experiment was performed on neutrophils isolated from 10 volunteers in their basal state; all experiments gave similar results). The phagocytosis index is the number of *C. albicans* phagocytosed by 100 neutrophils (for more details of the methods of phagocytosis see Ortega et al., 2005b). $^*p < 0.01$ vs. control, $^\bullet p < 0.01$ vs. results obtained after the incubation of neutrophils with the basal concentration of eHsp72 (ANOVA-Scheffe *F*-test)

NFkB.[2] This agrees with previous studies evaluating the effects of intense exercise (1 h of cycling at 70% VO_2 max) performed by sedentary women in which the increased chemotaxis of neutrophils was also mediated by eHsp72 via TLR-2 (together with $CD14^+$) and the intracellular signals ERK, PI3K, and NFkB, but independent of Ca^{2+} (Ortega et al., 2009). In the stimulation of neutrophil chemotaxis induced by a single bout of moderate exercise there also participates eHsp72, since the post-exercise concentrations stimulate in vitro the chemotactic capacity of neutrophils, and present a chemoattractant capacity that is even greater than that induced by the basal concentrations of the proteins (Figs. 4a and b). We can summarize by saying that the eHsp72 released during single bouts of moderate exercise

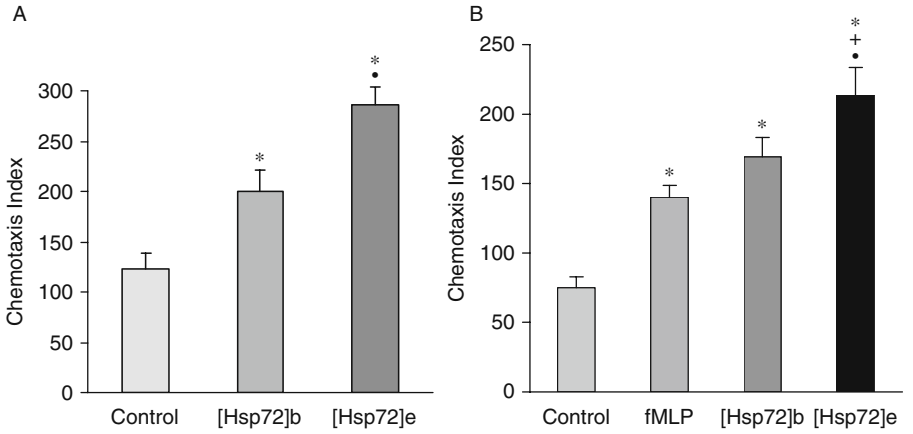

Figure 4. *Left part* (**a**) shows the in vitro effect on neutrophil chemotaxis of Hsp72 released into the blood following a single bout of moderate exercise (55% VO_2 max during 45 min on an ergometric cycle) performed by sedentary men. Neutrophils were incubated with Hsp72 at 0 (control), 5.12 ng/ml (basal concentration, [Hsp72]b), or 7.66 ng/ml (post-exercise concentration, [Hsp72]e) in the upper part of the Boyden chamber. In the lower part of the chamber, 10^{-5}MfMLP was added. *Right part* (**b**) shows the chemoattractant capacity of Hsp72. In this study, fMLP (10^{-5} M; a chemoattractant peptide), the basal concentration of Hsp72 ([Hsp72]b, 5.12 ng/ml), or the post-exercise concentration of Hsp72 ([Hsp72]e, 7.66 ng/ml) was put into the lower compartment of the Boyden chambers. Control values were those obtained only with Hank's medium (with no chemoattractant property). The chemotaxis index is the number of neutrophils counted at random (under 100× phase-contrast microscopy) in 16 fields of the lower face of the filter (for more details of the methods of chemotaxis see Ortega et al., 2009). Each column represents the mean ± SEM of 10 independent experiments performed in duplicate (each experiment was performed on neutrophils isolated from 10 volunteers in their basal state, all experiments gave similar results). $^*p < 0.01$ vs. control values; $^\bullet p < 0.01$ vs. values obtained after incubation of neutrophils with [Hsp72]b (ANOVA Scheffe *F*-test) in Fig. 4a. $^*P < 0.01$ vs. control values (Hank's medium). $^\bullet P < 0.01$ vs. [Hsp72]b values; $^+P < 0.01$ vs fMLP values (ANOVA-Scheffe *F*-test) in Fig. 4b

[2] Data presented in the 2nd Iberoamerican Congress on Neuroinmunomodulation (Madrid, Spain, 2007; Giraldo et al., p. 98 of abstract book) and VI Iberoamerican Congress of Sports Medicine (Sevilla, Spain, 2007). Giraldo, E. and Ortega, E. (2007). Archivos de Medicina del Deporte 24, 353 (Abstract).

performed by non-active men stimulates all the stages of the phagocytic process (i.e., chemotaxis, phagocytosis, and microbicidal activity), a process in which, at least, the TLR-2 are involved.

INTERACTION BETWEEN NE AND EHSP72 IN THE MODULATION OF NEUTROPHILS DURING MODERATE EXERCISE: MODEL OF CONJOINT ACTION OF NE AND EHSP72 DURING A POSSIBLE PATHOGEN CHALLENGE

So far, we have said that physical exercise, including at moderate intensity, releases stress hormones and proteins that can act as "danger signals" or "stress mediators" activating the innate immune responses, as has been found for NE, eHsp72, and the functional response of neutrophils. The "danger theory" (Matzinger, 1994, 1998) suggests that the immune activation involves schemes of recognition of "danger" and "no danger" molecules. There is evidence suggesting that Hsp72 molecules are candidates as danger signals during physical exercise stress (Fleshner et al., 2003), with NE having been posited as being a messenger of stress (Ortega et al., 2007) even prior to the release of Hsp72, with it participating in that release. Based on investigations conducted in our laboratory, we have proposed a model for the conjoint action of NE and eHsp72 in stimulating neutrophils during moderate exercise, and in their response to a possible pathogen challenge (Fig. 5).

The NE and eHsp72 released during moderate exercise can stimulate the movement of neutrophils, inducing in situations of physical exercise (and probably in other situations of stress) a greater capacity of movement towards exogenous chemotactic gradients produced by infective microorganisms, as well as attracting neutrophils to the sites where the protein itself can be released, sites which would include the foci of infections. There, at these inflammatory sites, NE and eHsp72 could again attract more phagocytes. In support of this hypothesis, it has recently been proposed that Hsp72 is released into the inflammatory sites (free or bound to other exogenous molecules) from the immune cells themselves, and even extravasating from the blood into those sites resulting in an enhanced immunological response (Asea, 2005). This model also agrees with the observation that subcutaneous injection of bacteria causes an increase in the concentration of Hsp72 in the skin (Campisi et al., 2003), and that the neutrophils themselves are a source of expression and production of extracellular Hsp72 during inflammatory processes in which these cells transmigrate through the endothelial cells of the blood vessels (Hennigan et al., 1999). In addition, there has been demonstrated an association between elevated eHsp72 concentrations after stress and reduced experimentally-induced inflammation (Campisi and Fleshner, 2003), as well as increased macrophage phagocytosis and accelerated wound closure (Kovalchin et al., 2006; Wang et al., 2006).

The interaction between NE and eHsp72 continues to be manifest in preliminary experiments whose surprising results indicate that the concentrations of both NE and eHsp72 released during single bouts of moderate exercise not only separately

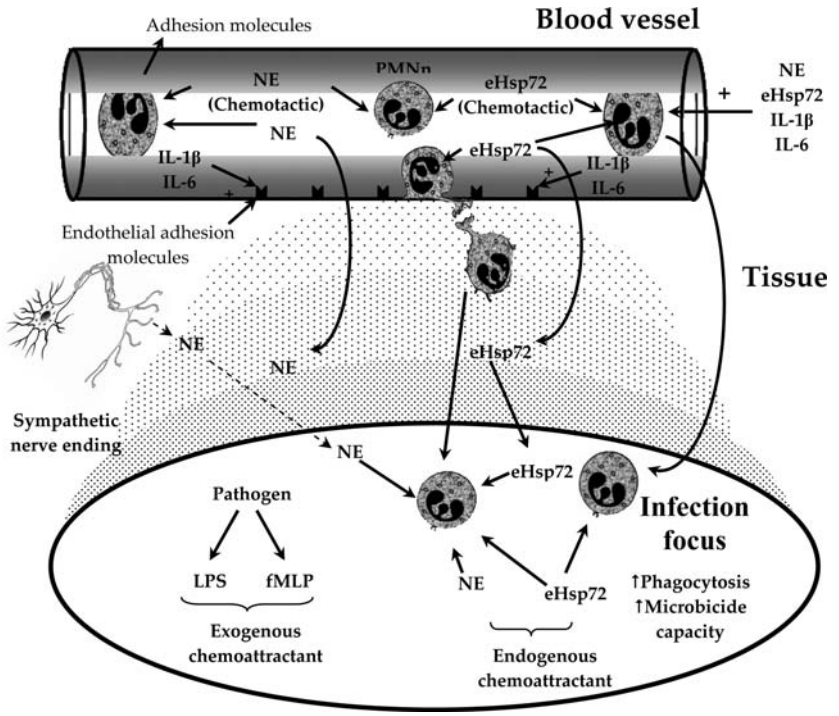

Figure 5. Model of the functional interaction between NE and eHsp72 in the stimulation of the phagocytic process of neutrophils. The model suggests that NE and eHsp72 systemically released into the bloodstream during moderate exercise stimulate the chemotactic capacity of neutrophils to move towards the source of infection along exogenous chemotactic gradients produced by infective pathogens, such as LPS or fMLP. In addition, these two "danger signals" could also participate in the systemic release of pro-inflammatory cytokines such as IL-1β or IL-6, which in turn would stimulate endothelial adhesion molecules and activate neutrophils to facilitate their extravasation and approach to the focus of inflammation. Also, both eHsp72 and NE (from the blood, leukocytes, nerve endings), would also act at the inflammatory focus as endogenous chemoattractants, recruiting more neutrophils to the site of inflammation. There they would finally activate the phagocytic and microbicidal capacity of the phagocytes, helping to combat and eliminate the infective pathogens

stimulate the chemotaxis capacity of neutrophils, but that in vitro this stimulation is greater when the two molecules act together. In addition, both the alpha- and the beta-adrenergic receptors participate in that stimulation, since blocking them with phentolamine or propranolol, respectively, impedes the conjoint stimulatory effect and even the stimulatory effect of eHsp72 alone.[3] We have also observed the novel participation of the adrenergic receptors in the stimulation by eHsp72 of neutrophils

[3] Data presented in the VI Iberoamerican Congress of Sports Medicine (Sevilla, Spain, 2007). Hinchado, M.D. and Ortega, E (2007). Archivos de Medicina del Deporte 24, 352 (Abstract).

(as well as the TLR-2 receptors) in in vitro trials evaluating the phagocytic and microbicidal capacity of these cells with the physiological concentrations of eHsp72 released during intense exercise sessions[4] (data in preparation for publication). This suggests that it also occurs at the concentrations released during single bouts of moderate exercise, as we are at present confirming in our laboratory (unpublished data).

CONCLUSION

It has been known for years that the catecholamines present an immunomodulatory capacity. Classically, they were ascribed an immunosuppressory role during situations of stress, since the first studies showed an inhibitory effect on the proliferative capacity of lymphocytes in vitro (Madden and Livnat, 1991). Nonetheless, the notion that catecholamines are always immunosuppressive has changed radically in recent years, with the observation that they can stimulate several aspects of the innate immune response at the physiological concentrations released during situations of stress. During physical exercise, these neurohormones may also mediate the effects of the stimulation of phagocytes, with a greater role for NE during moderate exercise, it being seen as a "stress mediator" (Ortega, 2003; Ortega et al., 2007). Also, recent investigations strongly suggest a close relationship between NE and eHsp72 in the activation of the innate immune response during situations of stress in general, and during exercise in particular, including that of moderate intensity. The fact that NE is involved, primarily through alpha-adrenergic receptors, as a signal inducing the release into the bloodstream of eHsp72 (Johnson et al., 2005) was one of the earliest pieces of evidence for the interaction between the two molecules in an organism's response to stress. Also, since Asea assigned the eHsp72's the role of "chaperokines" (Asea et al., 2000), their release during situations of stress in general and physical exercise in particular has accredited them as "danger signals" for the immune system (Fleshner et al., 2003), with an important role in stimulating the function of cells involved in the innate response during acute moderate exercise (Ortega et al., 2006).

Although it seems clear that NE (released quickly during situations of stress) is involved in the release of eHsp72 (released between 10 and 30 min after the onset of stress), it is still not very clear which are the "cellular sources" of Hsp72 nor the mechanism of their release. There seems to be an active release mechanism via a receptor-mediated exocytotic pathway involving exosomes and lipid rafts (reviewed by Asea, 2006). Bearing in mind the ubiquity and the sympathetic activation of Hsp72 release, it is starting to be accepted that Hsp72 would be released from any cells in tissues innervated by sympathetic neurons (reviewed by Johnson and Fleshner, 2006), or in response to systemic NE, for example in blood leukocytes.

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[4] Data presented in the VI Iberoamerican Congress of Sports Medicine (Sevilla, Spain, 2007). Giraldo, E. and Ortega, E (2007). Archivos de Medicina del Deporte 24, 352 (Abstract).

In addition, it is now well known that innate immune and/or antigen presenting cells, such as NK cells, neutrophils, monocytes/macrophages, and dendritic cells have receptors for NE (reviewed by Besedovsky and del Rey, 2007) and eHsp72, including several pattern recognition receptors (PRR's) such as TLR 2, 4, and 7 (reviewed by Whitham and Fortes, 2008). Since moderate exercise can stimulate the function of these cells in parallel with an elevated NE and eHsp72 release, and their post-exercise concentrations stimulate in vitro the function of innate cells (e.g., neutrophils), it is plausible to think that the interaction between NE and eHsp72 occurs both in the release of the two "danger signals" and in the conjoint action of the two molecules to stimulate the innate immune response during moderate exercise.

Nonetheless, it is still a matter of debate whether the in vitro effects on the immune cells induced by "free eHsp72" can be extrapolated to their effects in vivo. Some research groups think that the effect of "naked" eHsp72 in vivo is unlikely, due to its ability to chaperone antigens (i.e. LPS), thereby stimulating both innate and acquired immunity (Johnson and Fleshner, 2006; Whitham and Fortes, 2008). In our view, this debate in no way invalidates the more than possible in vivo stimulatory role of "free eHsp72" in the immune function during moderate exercise. There are two main reasons for this opinion:

1. If the eHsp72's are regarded as "danger signals" or "messengers of stress", they should also be able (as is NE) to activate (or pre-activate or "alert") the immune cells in the absence of any antigenic stimulus, i.e., prior to any pathogen challenge. (The effect of this activation is confirmed in vitro by discarding the possible role of LPS contamination). Additionally, the eHsp72's, chaperoning LPS, or other antigens in vivo, could help develop the immune response during exercise in individuals previously infected by a pathogen or at the start of infection.
2. Thus, the effects of the "free eHsp72" released during exercise or of chaperoning antigens must not be exclusive, being also able to contribute to the stress tolerance and protection of the immune cells (as well as others), particularly those involved in inflammatory processes, such as phagocytes, which after phagocytosing the antigen (even chaperoned by Hsp72) produce large amounts of reactive oxygen species.

The optimal level of exercise that improves, but does not impair or over-stimulate, an optimal immune function is not yet well known, especially in women, who have higher innate or "inflammatory markers" (also NE and eHsp72) in their basal state (Giraldo et al., 2008). A well controlled and regulated stimulation of the innate immune mechanisms during moderate exercise can help prevent infections, but over-stimulation of the innate and/or inflammatory response (sometimes during single or repetitive bouts of high intensity exercise) could also be harmful for people with inflammatory and/or autoimmune diseases. The modulation of the systemic or local pro-/anti-inflammatory cytokine balance during stress, including exercise, may suppress or potentiate autoimmune disease activity and/or progression, and catecholamines (Elenkov and Chrousos, 2002) and eHsp72 (Asea et al., 2000; Asea, 2005) are involved in this regulation. Although an association has been

found between survival and high levels of eHsp72 in patients with trauma (Pittet et al., 2002), elevated basal blood levels of eHsp72 have been also found in humans with different diseases, including inflammatory diseases such as atherosclerosis, Alzheimer's disease, or inflammatory bowel disease (Pockley et al., 2003; Pockley, 2002; reviewed by Johnson and Fleshner, 2006). Nevertheless, although reactivity to heat shock proteins has been associated with inflammatory diseases, recent evidence also suggests that the induction of self heat-protein immunoreactivity can attenuate autoimmunity and is anti-inflammatory (Pockley, 2003). Thus, an optimal level of "stress of life" is also very important (Prohászka and Füst, 2004). Prohászka and Füst propose that several kinds of stress and inflammation generate immune responses to Hsp, which are primary targets of autoimmunity and as such, are in need of protection by regulatory T cells (van Eden et al, 1998). In case of some disequilibrium in this protection, autoimmune and/or inflammatory diseases can emerge (Prohászka and Füst, 2004). In this context, the regular performance of moderate exercise leading to the release of Hsp72 could contribute to a good "training of the immune system", with particular importance in people who are increasingly sedentary and frequently subjected to psychological stress as a result of their "modern lifestyle". On the contrary, exercise performed at inappropriate intensities (irregular and high intensity) and/or in states of poor health may result in dysregulation in the immunophysiological mechanisms of these "danger signals", potentiating or exacerbating the disease. Thus, the roles of NE and eHsp72 in vivo may be different depending on the health status of the organism. Then, the conjoint action of NE and eHsp72 (depending on the moment of their release and the concentration reached) could facilitate innate immunity in a normal physiological state in healthy people, but might exacerbate inflammatory diseases in people suffering from them.

Better understanding the mechanisms underlying the improvement of the immune response and of the general state of health induced by exercise of moderate intensity, which may include the roles of NE and eHsp72, will also allow one to create pharmacological strategies to apply to individuals whose characteristics make it difficult for them to exercise. Also, understanding the type of intensity and timing best suited to performing physical exercise, according to each person's characteristics, will help avoid the possible undesirable effects of exercise, such as the exacerbation of autoimmune and/or inflammatory diseases.

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CHAPTER 20

MOLECULAR CHAPERONES AS MEDIATORS OF STRESS PROTECTIVE EFFECT OF PLANT ADAPTOGENS

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Abstract: The ability of plant adaptogens to enhance the "state of non-specific resistance" of an organism to stress by augmenting resistance to physical, biological, chemical and psychological stresses, and increasing concentration, performance and endurance during fatigue have placed it in a unique position among medicinal plants. However, the molecular mechanism by which plant adaptogens exerts its beneficial effects is thus far incompletely understood. This chapter focuses on recent advances in the understanding the molecular mechanism exerted by ADAPT-232 forte, a plant adaptogens consisting of a fixed combination of three extracts of *Eleutherococcus senticocus*, *Schisandra chinensis* and *Rhodiola rosea*. Our studies suggest that ADAPT-232 exerts its beneficial effect, in part, by a mechanism dependent on the upregulation of Hsp70 expression. A concise discussion of the effect of adaptogens on endurance and a comparison of hormetins and adaptogens will also be discussed

Keywords: Adaptogens; *Eleutherococcus senticocus*; heat shock proteins; hormesis; longevity; *Rhodiola rosea*; *Schisnadra chinesis*

Abbreviations: DAF-16, forkhead box O transcription factor protein; *daf-16*, forkhead box O transcription factor gene; HPA, hypothalamic-pituitary-adrenal; HSF-1, heat shock factor-1 protein; *hsf-1*, heat shock factor-1 gene; Hsp72, seventy kilo Dalton stress-inducible heat shock protein; IGF-I, insulin-like growth factor 1; JNK, c-Jun N-terminal kinase; JKK, c-Jun N-terminal kinase kinase; LD, lethal dose; NADPH, nicotinamide adenine dinucleotide phosphate; NOAEL, no observable adverse effect level; RNAi, RNA interference; TNF-α, tumor necrosis factor-alpha; TTE, time taken to exhaustion

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INTRODUCTION

Adaptogens are medicinal plants that enhance the "state of non-specific resistance" of an organism to stress, augmenting resistance to physical, biological, chemical and psychological stresses, and increasing concentration, performance and endurance during fatigue (Brekhman and Dardymov, 1969; Panossian, 2003, 2005; Panossian and Wagner, 2005; Olsson et al., 2008; Panossian and Wikman, 2008). The mechanism of action of adaptogens is partially associated with the hypothalamic-pituitary-adrenal (HPA) axis, a part of the stress system that also contributes to the nervous, cardiovascular, immune, gastrointestinal and endocrine systems (Panossian et al., 1999). Involvement of cortisol, nitric oxide, stress-activated protein kinase JNK and DAF-16/forkhead box O transcription factor in stress-protective effects of adaptogens was documented in several studies (Panossian et al., 1999, 2007; Wiegant et al., 2008a, b). However a role of molecular chaperones (heat shock proteins) in adaptogen-induced resistance to stress has to be clarified (Ip et al. 2001; Chiu and Ko, 2004; Wiegant et al., 2008a; Panossian et al., 2008).

Molecular chaperones are a group of proteins that promote the correct three-dimensional folding of proteins, prevent their aggregation and assist in re-folding of misfolded proteins, which are the main contributors to many devastating human diseases. The 70 kDa heat shock protein, Hsp72 plays a central role in the mechanism that rids the cell of stress induced misfolded or incompletely synthesized polypeptides that otherwise would interfere with normal cellular function, thereby playing a critical role in maintaining cellular homeostasis and in protecting the cell from stressful conditions and increase cell survival in the face of otherwise lethal cellular stress. In addition, Hsp72 may function as an endogenous "danger signal" for the immune system. The danger theory postulates that immune activation involves danger/non-danger molecular recognition schemas and suggests that innate immune cells are activated by danger signals that are derived from stressed or damaged self-proteins (Matzinger, 1998; Gallucci and Matzinger, 2001). It is now widely accepted that circulating serum Hsp72 fit this criteria (Asea, 2007, 2008). The model system which has emerged is that exposure to physical or psychological acute stressors stimulate the release of endogenous Hsp72 into the systemic circulation and that elevated Hsp72 functions to facilitate innate immunity in the presence of bacterial challenge (Febbraio and Koukoulas, 2000; Gonzalez and Manso, 2004; Asea, 2005; Fleshner and Johnson, 2005; Lancaster and Febbraio, 2005; Whitham and Fortes, 2008).

EFFECT OF ADAPTOGENS ON ENDURANCE: STUDIES OF ADAPT-232 FORTE

ADAPT-232 forte is a proprietary name of a fixed combination of three genuine (native) extracts of *Eleutherococcus senticocus* (Rupr. et Maxim) Harms root, *Schisandra chinensis* (Turzc) Baill., root, *Rhodiola rosea* L., root and vitamin B_5, characterized for the content of eleutherosides E and B (0.17%), schisandrin and

gamma-schisandrin (0.85%), salidroside (0.33%), tyrosol (0.07%), rosavin (0.37%), triandrin (0.01%) and calcium panthotenate (42.8%). Using a soft extract containing 24% of water and 2% of marker compound, we recently demonstrated that ADAPT-232 forte greatly potentiates the ability of mice to overcome exhaustive physical exercise (forced swimming with additional load) by a mechanism, in part, dependent on the significant increase in circulating serum Hsp72 levels (Panossian et al., 2008; Panossian and Wikman, 2008). Similar results were obtained in another study with ADAPT-232 without Vitamin B5 (Panossian et al., 2008; Panossian and Wikman, 2008). In this study, two series of experiments were carried out with mice taking ADAPT-232 forte in concentrations of 10, 30 and 60 mg/ml for 7 consecutive days followed by forced swimming test to exhaustion.

Our studies demonstrate that ADAPT-232 forte strongly augments the endurance of mice and increases the time taken to exhaustion (TTE) in a dose-dependent manner from 3.0±0.5 to 21.1±1.7 min, approximately a sevenfold increase (Figure 1). Circulating serum Hsp72 was measured both in normal and stressful conditions before and after the swimming test (Figure 2). Our data demonstrates that repeated administration of adaptogen dose-dependently increases the basal level of circulating serum Hsp72 in mice from 0.8–1.5 to 5.5–6.3 pg/ml (Figure 2; lines b–d). This effect

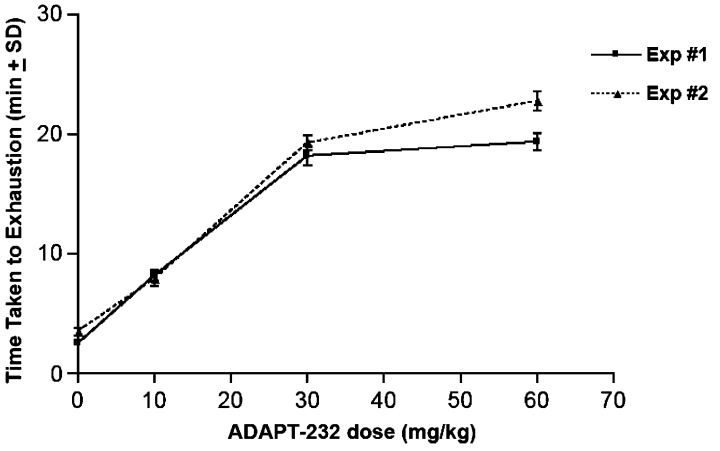

Figure 1. Plant adaptogen ADAPT-232 forte augments endurance of mice in a swimming model. ADAPT-232 forte was dissolved in the drinking water of 8–10 week-old female BALB/c mice (18.0±0.1 g) at various concentrations (0, 10, 30 and 60 mg/ml). Mice were allowed to drink freely for 7 consecutive days before the swimming test. Briefly, animals were placed into a transparent bucket filled with water at ambient temperature and allowed to swim for 30 min. After which animals were dried and a weight equivalent to 6% body weight was attached to the tail and animals placed back in the water bucket. The time taken to exhaustion (TTE) was recorded as the time taken at which the animal stopped swimming. Abscissa represents the ADAPT-232 concentration (mg/kg). Ordinate represents TTE (min ± SD). Data is the sum of two independently performed experiments with 5 mice per treatment group. * $p < 0.05$

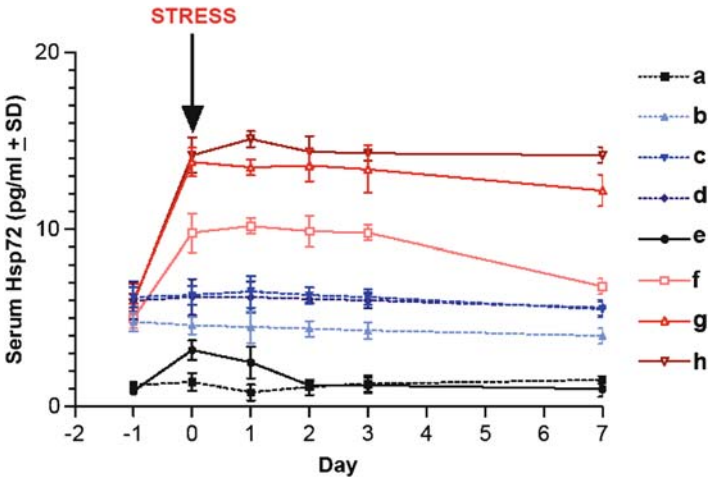

Figure 2. Plant adaptogen ADAPT-232 forte stimulates an increase in circulating serum Hsp72. ADAPT-232 forte was dissolved in the drinking water of 8–10 week-old female BALB/c mice (18.0±0.1 g) at various concentrations (0, 10, 30 and 60 mg/ml). Mice were allowed to drink freely for 7 consecutive days before the swimming test. Briefly, animals were placed into a transparent bucket filled with water at ambient temperature and allowed to swim for 30 min. After which animals were dried and a weight equivalent to 6% body weight was attached to the tail and animals placed back in the water bucket. The time taken to exhaustion (TTE) was recorded as the time taken at which the animal stopped swimming. Animals were then taken out of the water dried and placed into the respective cage and at various times blood was drawn and the concentration of circulating serum was measured using a modified Hsp70 sandwich ELISA. Data represents animals treated with, (**a**) Control, (**b**) ADAPT-232 forte (10 mg/ml), (**c**) ADAPT-232 forte (30 mg/ml), (**d**) ADAPT-232 forte (60 mg/ml), (**e**) Control + Stress, (**f**) ADAPT-232 forte (10 mg/ml) + Stress, (**g**) ADAPT-232 forte (30 mg/ml) + Stress, (**h**) ADAPT-232 forte (60 mg/ml) + Stress. Abscissa represents number of days. Ordinate represents the serum Hsp72 concentrations (pg/ml ± SD). Data is the sum of two independently performed experiments with 5 mice per treatment group. * $p < 0.05$

is significantly higher than the effect of both physical (swimming) and emotional stress combined 3.2±1.2 pg/ml (Figure 2; line e). The cumulative effect of stress and adaptogen was clearly observed in groups of animals treated with adaptogen after swimming to exhaustion (TTE), when circulating serum Hsp72 increases up to 15.1±1 pg/ml and remained at this level over 7 days. The data presented in this study demonstrate that the adaptogen, ADAPT-232 forte, strongly augments endurance of mice increasing the TTE in a dose dependent manner approximately sevenfold, by a mechanism, in part, dependent on the release of Hsp72 into systemic circulation.

These studies are significant because of current interest in the role of circulating serum Hsp72 as a danger signal and its biological significance to human physiology (Asea and De Maio, 2007). Indeed, there is now great interest in findings agents which can augment the increase in circulating Hsp72 for therapeutic gain in various human diseases. Basically, Hsp 72 inducers could have a therapeutic application in the treatment of neurodegenerative diseases, which may benefit

from increased chaperon expression to reduce the levels of misfolded or aggregated proteins that underlie the development of the pathological condition (Westerheide and Morimoto, 2005), while Hsp inhibitors may be of particular benefit for the treatment of cancer becaure tumor calls typically express high levels of Hsps than normal cells (Powers and Workman, 2007). A recent example is the compound STA-4783 (Synta Pharmaceuticals, Boston, MA), which has been shown to function, in part, by inducing the release of Hsp72 from tumors. In a double-blind, randomized, controlled Phase 2b melanoma study conducted in 21 centers in the US, patients treated with STA-4783, in combination with Paclitaxel, demonstrated significantly increased serum Hsp72 levels, decreased the extent of metastasis and increased the doubling of progression-free survival (Third International Melanoma Research Congress, Noordwijk, The Netherlands, September 2006). Studies are now ongoing to determine the efficacy of ADAPT-232 forte in various tumor animal models (Kaur et al., in preparation). The clinical significance of our observation can be associated with application of ADAPT-232 for the treatment of patients in the period of their reconvalescence from infectious diseases, when their immune system has to be supported and normal physiological functions recovered. It might be speculated that beneficial effect of ADAPT-232 on the recovery in patients suffering from infectious diseases is due to upregulation of Hsp 72 which in turn stimulates immune system and repairs denaturated proteins which are dramatically increased in acute phase of inflammation. Indeed, adjuvant therapy with ADAPT-232 has a positive effect on the recovery of patients by decreasing the duration of the acute phase of the illness, by increasing mental performance of patients in the rehabilitation period, and by improving their quality-of-life (Narimanyan et al., 2005).

In our experiments in mice, we demonstrated that adaptogens induces increase of serum Hsp72, regarded as a defense response to stress, and increase tolerance to stress (in our model combination of physical and emotional stresses). Our data suggest that increased tolerance to stress induced by adaptogen is associated with its stimulation of expression of circulating serum Hsp72 and particularly to Hsp72 production and release, which is known as mediator of stress response involved in reparation of proteins during physical load. Our working hypothesis is that adaptogens adapt (make less sensitive) the organism to stress, acting some-like low molecular weight "vaccines" or stress-mimetics inducing mild activation of stress system in order to cope more severe stress.

ADAPTIVE STRESS RESPONSE: EFFECT OF HORMETINS AND ADAPTOGENS

The adaptive stress response results in increased expression of heat shock proteins. Such a response sometimes is classified as hormetic response (Mattson et al., 2007). The initial term "hormesis" was defined as the generally-favorable biological responses to low exposures to toxins and other stressors (Calabrese, 2004). In this model at low doses there is a dose-dependent increase in beneficial biological

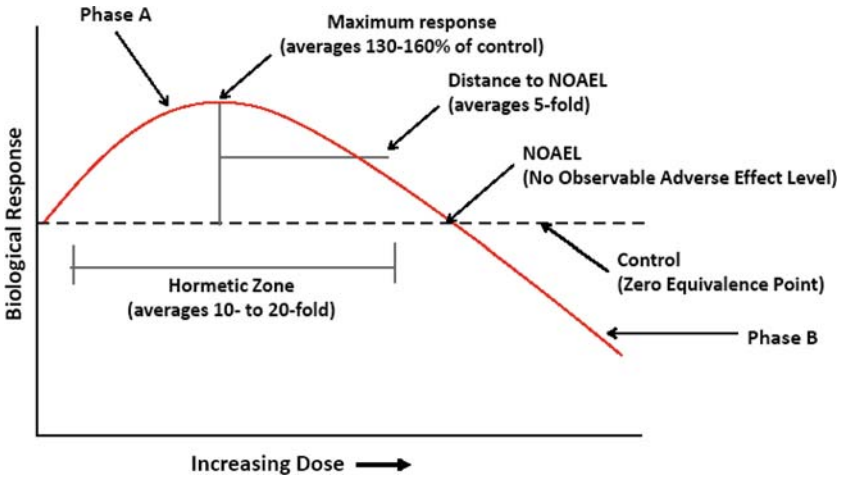

Figure 3. Characteristic dose-response curves for hermetics. Low dose of hormetins induces a dose-dependent increase in biological response until a maximum response is reached, approximately 130–160% of control values (Phase A). This hermetic zone is on average 10- to 20-fold concentration. After which there is a gradual decrease in biological response until it reaches the no observable adverse effect level (NOAEL). This is the same point at which the control values are found, and is known as the zero equivalence point. As the concentration continues to increase, there is now a significant decrease in biological response (Phase B). Abscissa represents the increasing dose of hermetins. Ordinate represents the biological response being measured

response until a maximum response is reached, approximately 130–160% of control values (Figure 3; Phase A). This hermetic zone is on average 10- to 20-fold concentration. After which there is a gradual decrease in biological response until it reaches the no observable adverse effect level (NOAEL). This is the same point at which the control values are found, and is known as the zero equivalence point. As the concentration continues to increase, there is now a significant decrease in biological response (Figure 3; Phase B). However, taking Paracelsus' paradigm into account that every substance is a poison, thus, all biological responses depend on the dose of the compound, in which case all compounds could be a hormetin, if it induces the adaptive response (Figure 3; Phase A). In that case, the term hormetin becomes ill-defined and only makes sense when Phase A is an obvious toxin. Adaptogens are substances which by definition induce an adaptive response phase A and do not have phase B (toxic effect) (Figure 3). In other words, adaptogens normally are not poisonous and cannot be considered as hormetins, which are generally toxic, e.g. dioxin, aflatoxins, pesticides, etc. (Tuomisto et al., 2006; Diaz et al., 2008; Hackenberger et al., 2008). Of course, in sufficiently high doses, except at dose levels far away from adaptive phase and conditions, like in experiments in C. elegance when worms are living all their life in the media rich of adaptogens their survival can be lower than in normal conditions (Wiegant et al., 2008a), similar as in experiments with oxygen when higher than normal concentration of oxygen can induce toxic effects.

The more definition of hormesis is a biphasic dose-response phenomenon characterized by low-dose stimulation and a high-dose inhibition (Calabrese, 2008a). In this model, hormetins exhibit a narrow dose range ($\leq$ 20-fold) before there is a significant decrease in biological response. However, adaptogens exhibit a wide response (> 1000-fold) before the zero equivalence point is reached (Figure 4). There is a tendency to expand the term of hormesis in "adaptive stress response" in general and which accounts for the beneficial effects non-toxic and generally recognized safe natural substances and botanicals (Mattson et al., 2007; Calabrese, 2008b, a; Mattson, 2008b, a). To our knowledge, to date, a dose-dependent reversal effect (biphasic dose response effect) has not been demonstrated for reservatrol, curcumin and sulforaphanes considered as "phytochemical neurohormetins" (Mattson et al., 2007). The effect of these compounds on signal transduction pathways involved in adaptive stress response does not mean that these substances are toxic in the amounts to which humans are normally exposed.

When comparisons of the adaptogen and hormesis concepts are made, sticking similarities and differences can be observed. Similarities are that both compounds stimulate an adaptive response probably by initiation of common mechanisms of adaptation to stress. The difference is their range to toxicity. Hormetins are generally toxic at low doses and in comparison to adaptogens exhibit very low ratio toxic dose/therapeutic dose. An example is sodium arsenite, which induces significant cell proliferation at concentrations below 0.5 μM, but inhibit cell growth at concentration of higher than 5 μM (Yang et al., 2007). Adaptogens by definition are innocuous substance with very wide therapeutic index. An example is the *Eleutherococcus* root

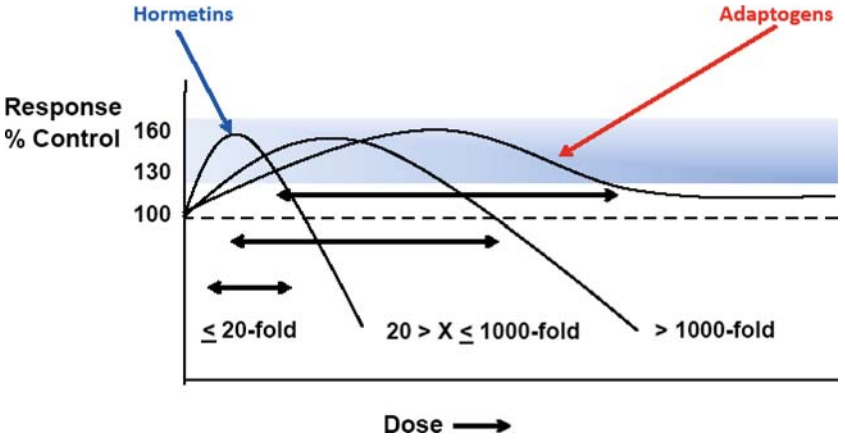

Figure 4. Characteristic biphasic dose-response curves of hermetic and adaptogens. Hormetins exhibit a narrow dose range ($\leq$ 20-fold) before there is a significant decrease in biological response. On the other hand, adaptogens exhibit a wide response (> 1000-fold) before the zero equivalence point is reached. Abscissa represents the increasing dose of hermetins or adaptogens. Ordinate represents the biological response being measured

extract which has an LD_{50} >3000 mg/kg. This is more than 1000-fold higher than the dose (3 mg/kg) increasing serum corticosteroids, working capacity and resistance to stress in rodents (Medon et al., 1981; Khasina et al., 1983). Similarly, the LD_{50} of Bryonia root extracts in mice is 1740 mg/kg, while stimulating effect on working capacity of mice was observed in the doses of 0.001–10 mg/kg (Pashinian et al., 1981).

Adaptogens exert an additional important beneficial effect of exhibited detoxifying effects. An example is the *Eleutherococcus senticosus* extract, which when injected at 20 ml/kg, i.p., into guinea pigs treated with the anti-tumor rubromycin C results in better tolerance to this toxic antibiotic (Goldberg et al., 1971). Combined use of the alkylating antitumor agents Thio-TEPA (Tsyrlina, 1965), Dopan (Tsyrlina, 1965), cyclophosphan (Monakhov, 1965, 1967b, a), ethymidine (Monakhov, 1967b, a), benzo-TEPA (Monakhov, 1967b, a), and sarcolysin (Stukov, 1966), taken with oral administration of *E. senticosus* liquid extract significantly decreased the toxicity of these anticancer agents and improved their antitumor effect (Wagner and Proksch, 1985). The toxic effect of subcutaneous injections of chlorofos (an organochlorophosphate cholinesterase inhibitor that is used as an insecticide for the control of flies and roaches, and in anthelmintic compositions for animals) was decreased by *E. senticosus* extract (Elkin, 1972). In a similar fashion, hemic hypoxia induced in mice by $NaNO_2$, and tissue hypoxia induced by malonic acid were reduced by treatment with the *E. senticosus* extract (Mikhailova and Fruentov, 1972). In combination with either cytarabine (a chemotherapy agent) or N6(△2-isopentenyl)-adenosine, aqueous extracts of *E. senticosus* gave additive antiproliferative effects against L1210 leukemia cells in vitro (Hacker and Medon, 1984; Wagner et al., 1985; Wagner and Proksch, 1985).

When a novel category or concept like "hormetic substance" is introduced there is often the tendency to apply it in a large range of compounds as possible. However, this could lead to an overuse of the concept which will "dilute" it and make it less precise as in the case of adaptogens (Wiegant et al., 2008a; b). In addition, it should be noted that hormetic (dose-dependent biphasic effects) data demonstrated in simple organisms might not be reproducible in humans, which are more complicated and have evolutionally advance defense systems in response to harmful toxins (Wiegant et al., 2008a; b). Finally, for adaptogens most important and valuable is their stress-protective effect and the ability to increase tolerance to stress, but not their ability to exert harmful effect at artificially high doses.

ROLE OF HEAT SHOCK PROTEINS

Schisandrin B is one of the active constituents of ADAPT-232. In a study by Ip and colleagues, pre-treatment of mice with *Schisandrin B* provided complete protection from hepatic apoptosis induced by injection of D-galactosamine sensitized mice with TNF-α (Ip et al., 2001). These authors also observed a concomitant increase in hepatic Hsp70 level in a dose-dependent manner. However, the relevance

of Schisandrin B-induced increase in hepatic Hsp70 expression in the prevention of TNF-α-mediated hepatic apoptosis was not determined (Ip et al., 2001).

A recent study demonstrated that increased expression of Hsp25 and Hsp70 contributed, in part, to cardioprotection afforded by *Schisandrin B* pre-treatment against myocardial ischemia-reperfusion injury in rats (Chiu and Ko, 2004). It was demonstrated that pre-treatment of rats with *Schisandrin B* (1.2 mmol/kg) resulted in the increase in Hsp25 and Hsp70 in rat heart, reaching a maximum on day 2 and 3 post treatment. The fact that exposure of rats to heat shock resulted in similar cardioprotection and increased Hsp25 and Hsp70, strongly suggests that some of the beneficial effects of *Schisandrin B* is transduced by its ability to augment the expression of heat shock proteins (Chiu and Ko, 2004).

The expression of the constitutively expressed heat shock protein, Hsc70, was recently found to be significantly elevated in blood mononuclear cells, in the left heart ventricle and in the hippocampus of male Wistar rats after 7 days administration of ADAPT-232 (Panossian and Wikman, 2008). Interesting, the expression Hsp27, Hsc70, Hsp70 or Hsp90 was not upregulated in isolated hepatoma cells exposed to *Rhodiola* and *Eleutherococcus* (two other ingredients of ADAPT-232). Instead, expression of the small heat shock protein, Hsp32 (heme oxygenase-1) was observed (Wiegant et al., 2008a). The reason for this discrepancy is currently unknown. Hsp32 or heme oxygenase-1, is the inducible isoform of heme oxygenase that catalyzes the NADPH, oxygen, and cytochrome P450 reductase-dependent oxidation of heme to carbon monoxide, ferrous iron and biliverdin which is rapidly reduced to bilirubin. Hsp32 has been demonstrated to contribute to repair and protective mechanisms at the synapse after various stressors (Bechtold and Brown, 2000; Bechtold et al., 2000).

Studies from the Sherman laboratory have demonstrated that elevated levels of intracellular Hsp70 inhibit a signal transduction pathway leading to programmed cell death by preventing the activation of stress-induced activation of c-Jun N-terminal kinase (JNK), a very early stage of apoptotic process (Gabai et al., 1997; 1998). These finding provides the basis for the anti-apoptotic activity of Hsp72 and might explain increased stress sensitivity of aged cells in which compromised inducibility of Hsp72 leads to a loss of control of JNK activation by stresses and subsequently to a higher rate of apoptotic death (Gabai et al., 1998). Aged organisms exhibit a greatly decreased ability to induce the major heat shock protein, Hsp72, in response to stresses, a phenomenon that was observed in cell cultures (Heydari et al., 1994). Hsp72 was shown to protect cells from a variety of stresses. The protective function of Hsp72 has been commonly ascribed to its chaperoning ability. However, the ability of Hsp72 to inhibit JNK, an essential component of the heat-induced apoptotic pathway has begun to be addressed (Gabai et al., 1997; Volloch et al., 1998; Gabai et al., 2002). This group also demonstrated that the overexpression of Hsp72 or Hsc73 protected cells from heat shock-induced cell death. This required the chaperone function of Hsp72 since Hsp72 mutant proteins did not prevent procaspase processing or provide protection from apoptosis. In addition, JNK activation was inhibited by both Hsp72 and Hsc73 and by each of the Hsp72 domain mutant

proteins. The authors interpreted the data to suggest that the chaperoning activity of Hsp72 is not required for inhibition of JNK activation, and JNK inhibition is not sufficient for the prevention of apoptosis (Mosser et al., 2000).

Recently, we demonstrated that adaptogens, including *Schisnadra chinesis and Rhodiola rosea*, inhibits stress-induced JNK expression in rabbits (Panossian et al., 2007). These results are in line with other publications related to the effect of adaptogens on Hsp72 and DAF-16/forkhead transcription factor. DAF-16, a FOXO-family transcription factor, influences the rate of ageing of *Caenorhabditis elegans* in response to insulin/insulin-like growth factor 1 (IGF-I) signalling. Studies by Morley and Morimoto confirm data suggesting that the transcriptional activator heat shock factor 1 (HSF-1) regulates stress resistance genes and DAF-16/forkhead transcription

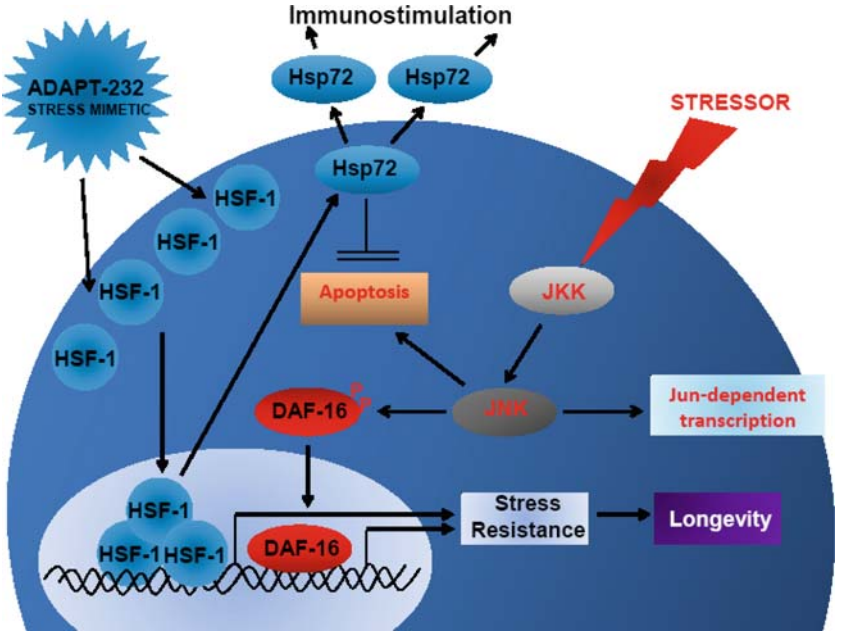

Figure 5. Schematic representation for the hypothetical molecular mechanism by which ADAPT-232 induces stress resistance and longevity. In the absence or presence of harmful stressful stimuli (*red lightning bolt*), ADAPT-232 (*blue star*) induces the activation of cytoplasmic HSF-1 (*blue circles*), which trimerizes and gains a nuclear translocation signal. In the nucleus (*light blue oval*), HSF-1 transcribes the synthesis of heat shock proteins (e.g., Hsp72; *blue oval*) which are transported to the extracellular milieu and exerts immunostimulatory effects on the hosts' immune system. Inside the cell (*blue half circle*), increased Hsp72 expression inhibits detrimental signal transduction cascades activated by stress, e.g., JNK (*dark grey oval*)-induced apoptosis (*brown rectangle*) activated by JKK (*light grey oval*). This allows some of the more favorable functions of JNK to be initiated, including Jun-dependent transcription (*blue rectangle*) and the phosphorylation cytoplasmic DAF-16/forkhead transcription factor (*red oval*), which then translocates to the nucleus and synthesizes additional proteins to confer stress-resistance (*blue rectangle*) and increased longevity (*purple rectangle*)

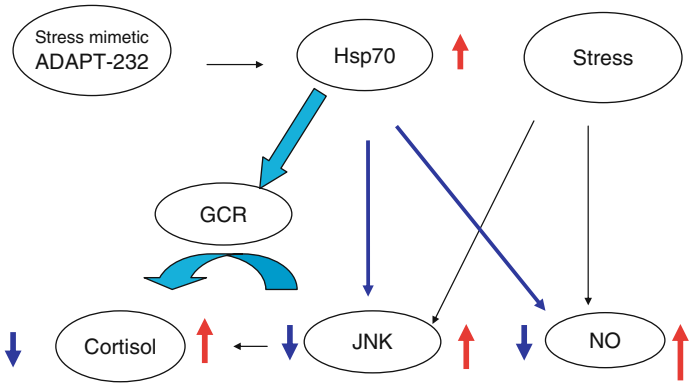

Figure 6. Schematic representation of stress induced activation (*red arrows*) of mediators of stress response and their interactions and anti-stress effect of ADAPT-232: upregulation is shown by *red arrow*, down-regulation – by *blue arrow*

factor function in a common pathway to regulate longevity (Morley and Morimoto, 2004). Using the *Caenorhabditis elegans* model, these authors demonstrated that RNAi for either *hsf-1* or *daf-16* genes shortened wild-type life span by 23 and 27%, respectively, and that combined silencing of both *hsf-1* or *daf-16* genes did not result in a further decrease in life span (Morley and Morimoto, 2004).

Based on data from our lab and published studies, our hypothetical model proposes that ADAPT-232 forte exerts its beneficial effects *via* the stimulation of the stress response (Figure 5) and interacts with other mediators of stress response (Figure 6). In the absence or presence of harmful stressful stimuli, ADAPT-232 induces the activation of cytoplasmic HSF-1, which trimerizes and gains a nuclear translocation signal. In the nucleus, HSF-1 transcribes the synthesis of heat shock proteins (e.g., Hsp72) which are transported to the extracellular milieu and exerts immunostimulatory effects on the hosts' immune system. Inside the cell, increased Hsp72 expression inhibits detrimental signal transduction cascades activated by stress, e.g., JNK-induced apoptosis activated by JKK. This allows some of the more favorable functions of JNK to be initiated, including Jun-dependent transcription and the phosphorylation cytoplasmic DAF-16/forkhead transcription factor, which then translocates to the nucleus and synthesizes additional proteins to confer stress-resistance and increased longevity (Figure 5).

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CHAPTER 21

BIOCHEMICAL CHANGES IN RESPONSE TO INTENSIVE RESISTANCE EXERCISE TRAINING IN THE ELDERLY

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Abstract: Sarcopenia, the age-related loss of muscle mass and muscle strength, is closely related to inflammatory processes and seems to be aggravated by concomitant age-related alterations in cell-protecting mechanisms involving heat shock proteins (HSP). The most effective modality to counter sarcopenia is intensive resistance exercise, which also induces an inflammatory reaction and influences Hsp70 expression in peripheral blood mononuclear cells (PBMC) and several tissues. In this review we focus on the influence of physical exercise on the HSP-expression during aging. Following a systematic literature search it can be concluded that there is very limited information available at this moment. Animal studies have described a blunted exercise-induced HSP response in the skeletal muscle, heart and liver of older rodents compared to younger ones. The human studies have shown that physical exercise lowers the basal Hsp70 expression in PBMC, probably by reducing the low-grade inflammation and the oxidative stress. A 6-weeks intensive resistance training program in elderly persons improves the heat-induced Hsp70 expression in PBMC, a phenomenon which might be related to a better cellular protection during stressful situations. More research is warranted in this domain, especially involving elderly persons in different clinical conditions and exploring the effects of different exercise schedules

Keywords: Sarcopenia; cytokines; heat shock proteins; physical exercise; elderly inflammation

Abbreviations: AGE, advanced glycation end products; HSF1, heat shock factor 1; HSP, heat shock protein; IGF-1, insulin-like growth factor-1; IL-6, interleukin; NO, nitric oxide; PBMC, peripheral blood mononuclear cell; RAGE, receptor for AGE; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha

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INTRODUCTION

During ageing significant alterations occur in the skeletal muscle. A well known phenomenon is the age-related muscle atrophy and strength loss, defined as sarcopenia (Rosenberg, 1997). The process starts at the age of 30–40 years and progresses insidiously at an average rate of 1% loss of strength per year (Marcell, 2003). Above the age of 70 years the loss rate increases up to more than 3% per year (Aniansson et al., 1992). Due to sarcopenia, activities of daily living in elderly persons necessitate efforts close to their maximal strength and loss of independency can occur (Hortobagyi et al., 2003). Sarcopenia-induced muscle weakness is, therefore, a typical characteristic of frailty, one of the major geriatric syndromes (Fried et al., 2001). Sarcopenia is extensively documented in the literature and belongs to the hot topics within the geriatric and gerontological research domains (Morley, 2004). The total cost of health care directly induced by sarcopenia in the United States of America for the year 2000 was estimated at 18.5 billion dollar (Janssen et al., 2004). It is to be expected that, given the worldwide ageing of the population, the cost will further increase, not only in industrialized, but also in developing countries.

FACTORS CONTRIBUTING TO SARCOPENIA

The underlying mechanism of sarcopenia is not yet completely understood. Given the high variability of sarcopenia, several factors will contribute to the process. As shown in Table 1, the factors identified can be subdivided into decreased anabolic and increased catabolic processes, for both of which endogenous and exogenous factors can be recognized. Remarkably, several factors are tightly related to inflammatory processes.

At the level of the muscle itself, sarcopenia is characterized by loss of muscle fibers and atrophy of the remaining muscle cells (Lexell et al., 1988). This phenomenon is strongly related to increased levels of inflammatory markers in the blood circulation (Visser et al., 2002; Cesari et al., 2004), especially interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α), which are known to have cytotoxic and proteolytic properties (Mackinnon, 1992; Gabay and Kushner, 1999). In fact, during normal aging circulating levels of inflammatory cytokines such as IL-6 and TNF-α become slightly elevated (Hager et al., 1994), a phenomenon corresponding to a chronic low-grade inflammatory profile (Krabbe et al., 2004). The exact mechanisms by which inflammatory cytokines promote myofibrillar proteolysis are not yet completely understood. Recent insights revealed an up-regulation of the ubiquitin-proteasome pathway and calcium-activated pathway of calpains induced by TNF-α, and probably also other cytokines (IL-1 or IL-6), and thus inducing muscle protein breakdown (Mitch and Goldberg, 1996; Argiles et al., 2000; Muscat and Dressel, 2000; Zoico and Roubenoff, 2002).

Muscle weakness due to sarcopenia is more important than can be explained by atrophy alone. The atrophy of type-2 (fast twitch) muscle fibers seems to be more

Table 1. Factors contributing to sarcopenia

Metabolic pathway	Type	Factor	References*
↓ Anabolism	Endogenous	↓ Hormonal stimulation (growth hormone, IGF-1, testosterone, oestrogen)	Carter et al. (2002), Grounds, (2002), and Payette et al. (2003)
		Loss of motorneurones, denervation of muscle fibres	Doherty et al. (1993) and Vandervoort (2002)
		↑ Non-contractile tissue in muscle	Overend et al. (1992) and Kent-Braun et al. (2000)
	Exogenous	↓ Physical activity	Doherty (2003) and Roubenoff (2003)
		Bed rest, immobilisation	Bloomfield (1997)
		Malnutrition	Morley (2001)
↑ Catabolism	Endogenous	↑ Basal inflammatory profile (IL-6, TNF-α)	Hager et al. (1994) and Ershler and Keller (2000)
	Exogenous	Stress-induced inflammation: life events, depression	Lutgendorf et al. (1999) and Sutherland et al. (2003)
		Disease	Ershler and Keller (2000), Giordano et al. (2003) and Jagoe and Engelen (2003)

*Non-exhaustive references.

important than the loss of type-1 (slow twitch) fibers, which might explain the important loss of muscle strength and explosivity (muscle power, the capacity to generate a high force in a short time) due to sarcopenia (Lexell et al., 1988). In fact, the absolute loss of explosivity with ageing is even more important than the loss of maximal strength (Lauretani et al., 2003), indicating that contraction-speed is also impaired due to sarcopenia. Interestingly, when normalized to cell size, the contractile strength and velocity of isolated muscle fibers are not significantly affected by ageing (Trappe et al., 2003). Supplementary loss of muscle contractile properties might be due to age-related alterations in the connective tissues surrounding the muscle fibers (endomysium, perimysium and epimysium). Less described in the context of sarcopenia is the age-related augmentation of the proportion of non-contractile tissue in the muscle (Kent-Braun et al., 2000). Besides proliferation of intra-muscular fat-tissue (Overend et al., 1992; Kent-Braun et al., 2000), the formation of cross-links between collagen molecules leads to profound changes in composition of the muscle-tendon complex as well as its mechanical properties (Avery and Bailey, 2005). Non-enzymatic alterations of the extracellular matrix, among which accumulation of Advanced Glycation End products (AGE, mediated by condensation of a reducing sugar with an amino group) (Monnier et al., 2005) are related to ageing and lead to permanent cross-links. These processes are responsible for an increasing proportion of insoluble extracellular matrix and thickening of the tissues, as well as increasing mechanical stiffness and loss of elasticity (Kjaer, 2004; Avery and Bailey, 2005; Monnier et al., 2005). Interaction of AGE with the receptor for AGE (RAGE) causes activation of intracellular signaling, gene expression, and production of IL-6, TNF-α and free radicals (Ahmed and Thornalley, 2007). The proteolytic activity of these inflammatory processes makes the collagen more vulnerable and increases sarcopenia. Moreover, inflammatory processes involving TNF-α can, by increasing the cellular production of reactive oxygen species (ROS) and nitric oxide (NO), depress muscle contractibility, thus inducing supplementary weakness (for review Zoico and Roubenoff, 2002).

SARCOPENIA AND ALTERED CYTOPROTECTIVE MECHANISMS

The aforementioned inflammatory processes related to sarcopenia (both systemic and at the tissue level) are thought to be intensified by a concomitant age-related decline in cellular protection mechanisms, i.e. heat shock protein (HSP) expression. In fact, HSP are expressed during stress-situations (e.g. hyperthermia, oxidative stress, infection) and protect the cellular integrity by acting as “chaperones” for intracellular proteins. Several HSP families are identified, which can be classified according to their molecular weight. Recently, a comprehensive classification based upon the HUGO Gene Nomenclature has been proposed, including HspA, HspB, HspC, HspD, HspH and DNAJ (Hightower et al., 2008). Especially the “Hsp70 chaperoning machine” (HspA-family) is the most inducible by stress (Vos et al., 2008). Alterations in Hsp70 expression are thought to be involved in age-related

dysfunctions such as thermoregulation and sarcopenia (Horowitz and Robinson, 2007; Lee et al., 2007).

An established method to study intracellular stress-induced HSP-expression is by exposing the cell to a heat shock (exposure to temperatures above 40°C). Under these conditions HSP transcription is up regulated via heat shock factor 1 (HSF1). It has been shown that heat-induced Hsp70 expression is significantly attenuated in peripheral blood mononuclear cells of older individuals (Rao et al., 1999; Njemini et al., 2002; Singh et al., 2006). Moreover, several animal studies demonstrated lower levels of Hsp60 and Hsp70 in skeletal muscle (Doran et al., 2007) as well as a lower stress-induced expression of Hsp70 in various tissues (for review (Lee et al., 2007)). Also, it is assumed that the promoter for the *hsp* gene has a responsive element both for HSF1 and for the signal transducers of inflammatory cytokines. Several reports have shown that during inflammation, the expression of Hsp70, both intracellular (Njemini et al., 2003b) and in serum (Njemini et al., 2003a; 2004), is related with circulating IL-6 and TNF-α in elderly persons. Also in the skeletal muscle itself, the expression of chaperones belonging to the Hsp70-family is up regulated when exposed to elevated levels of IL-6 (Febbraio et al., 2002b).

In fact, when applied separately, both heat-shock and inflammatory cytokines have a stimulating effect on HSP production. But when applied together, they appear to decrease each other's stimulating effect. Thus, it can be supposed that a constant, low-grade inflammatory profile in the elderly, characterized by slight elevations in circulating IL-6 and TNF-α, might negatively interfere with the induction of HSP during stressful conditions. Under these circumstances the cellular protection might be reduced and thus precipitate loss of muscle mass and muscle strength, a phenomenon seen during acute infections (Bautmans et al., 2005a), surgery and trauma in elderly patients.

The role of extracellular Hsp70 is not yet completely understood. It has been shown that the presence of extracellular Hsp70 can have a protective effect against necrotic cell death of smooth muscle cells (Johnson and Tytell, 1993) and against apoptosis of motor neurons (Robinson et al., 2005). From these insights in can be assumed that circulating Hsp70 plays a protective role against conditions related to inflammation. Recently, we have described the interference between circulating levels of IL-6 and Hsp70 on muscle endurance in elderly nursing home residents (Bautmans et al., 2008). In fact, subjects with both high serum levels of IL-6 and Hsp70 showed significantly worse muscle endurance compared to those with high IL-6 and low Hsp70. It has been reported that in the absence of serious inflammatory conditions, low levels of serum Hsp70 are associated to successful biological aging (Terry et al., 2006) and might reflect a strong anti-inflammatory status of the individuals' immune system (Franceschi et al., 2007). In our study, residents with acute conditions were excluded. In such situation, the signaling function of IL-6 is probably less inflammation-related. In fact, cytokines are also involved in the healing process and the down regulation of the acute phase in the resolution of inflammation (Kushner, 1998) and it has been suggested that IL-6 can exert both inflammatory and anti-inflammatory signaling (Tilg et al., 1997). High levels of serum Hsp70,

on the other hand, can be seen as reflecting higher and less controlled low grade inflammatory activity (Njemini et al., 2004), with detrimental effects on muscle cells.

RESISTANCE EXERCISE TO COUNTER SARCOPENIA

There exists a large body of evidence that physical exercise, particularly intensive strength training, can improve muscle strength in elderly persons, thus countering sarcopenia-related muscle weakness (Latham et al., 2003). Important strength gains (up to >100% in 6–9 weeks) can be obtained following strength training in the elderly, even in subjects aged 90 years and older (Fiatarone et al., 1990). The rapid strength gains (already in the first 4–6 weeks) are explained by increases in voluntary muscle activation; after prolonged training muscle hypertrophy becomes measurable (Hakkinen et al., 2000; Macaluso and Vito, 2004; Reeves et al., 2004).

Acute biochemical changes occur following intensive physical exercise, characterized by the release of signaling molecules and proteins, and a cascade of cellular responses (for review Pedersen and Hoffman-Goetz, 2000 and Coffey and Hawley, 2007), from which several are similar to the processes related to sarcopenia. In fact, physical exercise provokes an inflammatory reaction with the release of inflammatory cytokines, especially IL-6, into the blood circulation (Pedersen et al., 2001). In this context, IL-6 is mainly released from the contracting muscles (Ostrowski et al., 1998) and would exert a different function from that seen during e.g. acute infections, rather acting as a "myokine" and signaling the need for endogenous glucose production (Pedersen et al., 2001). Also enhanced HSP expression following physical exercise has been described in the muscle itself (Liu et al., 2000), as well as in peripheral blood mononuclear cells (Fehrenbach et al., 2000b; Whitham et al., 2004) and in the serum (Walsh et al., 2001; Febbraio et al., 2002a) of young individuals. These acute phase responses seem to play an essential role in the adaptation of the muscle following exercise. Possibly, the release of cytokines activates satellite cells in the muscle (Kadi et al., 2005) and the accumulation of HSP protects the muscle cells against the exercise-induced elevation of oxidative stress (McArdle et al., 2002). Moreover, it is assumed that Hsp70 can play a role in muscle hypertrophy following strength training (Kilgore et al., 1998). The activators for the HSP-response to exercise are probably multifactorial (for review Kregel, 2002). In fact, already within five minutes of a warm-up exercise of moderate intensity, the muscular temperature rises with 2°C (Saltin et al. 1968) and when exercising for prolonged periods, temperatures inside large muscles above 39°C have been described for humans and up to 44°C in rats (Brooks et al., 1971; Febbraio, 1996). Such elevated temperatures are known to be a powerful stimulus for Hsp70 (Njemini et al., 2002). On the other hand, since in vivo infusion of IL-6 activates intramuscular *hsp72* gene expression (Febbraio et al., 2002b), it can be assumed that exercise-induced accumulation of IL-6 is also a major contributor. Although studies are less abundant, elderly persons equally seem to be able to respond to physical stress by a significant increase of IL-6 in the blood circulation (Bruunsgaard and Pedersen, 2000; Toft et al., 2002; Bautmans et al., 2005b). It has been described, however, that acute eccentric exercise

induces a smaller systemic response in the aged (Toft et al., 2002) and did not elicit significant accumulation of IL-6 transcripts in the muscle of older subjects contrary to young ones (Hamada et al., 2005).

HSP-RESPONSE TO PHYSICAL EXERCISE IN THE ELDERLY

In this section, we will focus on experimental trials targeting the HSP-response to physical exercise in the elderly. Therefore, the bibliographic databases PubMed and Web of Science were systematically screened (Appendix) and the relevant articles found are reported and discussed.

ANIMAL STUDIES

Twelve animal-studies were identified and are summarized in Table 2. All investigations were performed on mice or rats and reported exercise-induced HSP-expression either in the skeletal muscle, the heart or the liver. The exercise stimuli applied consisted in treadmill running (endurance exercise) (Kregel and Moseley, 1996; Naito et al., 2001; Demirel et al., 2003; Starnes et al., 2003, 2005; Rinaldi et al., 2006; Huffman et al., 2008; Kayani et al., 2008) or electrical muscle stimulation (resistance exercise) (Vasilaki et al., 2002, 2003; Murlasits et al., 2006; Vasilaki et al., 2006).

Overall, a blunted HSP content, both at rest and following exercise, is reported in the tissues of the older animals compared to the young or middle-aged. In several studies, a significant exercise-induced increase in HSP-levels was detected in the older animals, although in a lower proportional change compared to young ones. Interestingly, Naito et al. (2001) demonstrated that in old rats the exercise-induced Hsp72 expression was similar to that seen in middle-aged animals for the high-oxidative (slow-twitch fibers) muscles, but lower for the muscles containing mainly fast-twitch fibers. Also, muscles containing many slow-twitch fibers showed higher resting HSP-levels compared to fast-twitch muscles, a phenomenon also reported by other investigators (Locke et al., 1991). Moreover, oestrogen plays a role in the expression of HSP, especially in heart. In fact, female animals show higher levels of Hsp72 in the heart compared to male, and this level decreases after ovariectomy (Voss et al., 2003). On the other hand, oestrogen seems to inhibit exercise-induced Hsp70 expression in rat skeletal muscle (Paroo et al., 2002), and stress-induced Hsp72 in rat pituitary gland, mesenteric lymph nodes and liver, but not in adrenal gland, spleen or heart (Paroo et al., 2002). Only 2 of the 12 studies we have found used female older animals (Naito et al., 2001; Vasilaki et al., 2002). At this moment, it remains unclear whether the gender ratio might have influenced the results of the animal studies. Interestingly, no age-differences were observed for exercise-induced responses of HSF1 protein and activation and HSP-mRNA, suggesting that the blunted HSP-response following exercise is not caused by reduced HSF1 availability or capacity to make mRNA in response to exercise.

Table 2. Studies describing the influence of physical exercise on HSP-expression in elderly animals

References	Population	Type of exercise	Main outcome	Results
Demirel et al. (2003)	Male Fischer-344 rats, 6 (Y) or 24 (O) mo randomized to control, exercise, hyperthermia	–2 d treadmill running (1 h/d, 75% VO2Max). –Hyperthermia, 15 min at 41°C (colonic temperature), using a heating blanket.	Myocardial –Hsp72 expression –HSF1 protein and activation –Hsp72-mRNA	–Lower ↑ myocardial Hsp72 after exercise and hyperthermia in O compared to Y –No difference between Y and O for HSF-1 protein, HSF-1 activation, and Hsp72-mRNA
Huffman et al. (2008)	82 Male C57BL/6 mice, aged 9 weeks, divided in 6 groups (young control, sedentary, exercise, exercise, 9%CR, exercise+9%CR, 18%CR)	24 wk treadmill running 5 d/wk (max 1 h)	–Hsp25, Hsp70 and Hsp70i in liver and skeletal muscle	–↑Hsp25 and Hsp70i in muscle of runners –↑Hsp25 in liver of runners+9%CR compared to sedentary
Kayani et al. (2008)	12–14 mo old (Y) and old 24 mo old (O) male C57BL6/J mice (*N*=96) divided in 5 groups (Y, O, Y training 10 wk, O training 10 wk, Y training 12 mo)	Treadmill running 15 m/min for 15 min 3 d/wk	–Whole M. Quadriceps Hsp70 content –Whole M. Gastrocnemius Hsp70-mRNA content	–↑Hsp70 and ↓Hsp70m-RNA in untrained O compared to untrained Y, differences not observed in trained O
Kregel and Moseley (1996)	12mo (A) and 24mo (O) old male Fischer 344 rats	3 groups: –Control –Passive heating (until corporal T=41°C) –Exertional heating (treadmill run until corporal T=41°C)	–Hsp72 in liver	–↑ Hsp72 in liver following –Heat (+192%l) in A, not in O –Exercise in A (+292%) and in O (+232%)
Murlasits et al. (2006)	Male (*N*=19) male Fisher344XBN F1 rats, 10 Y (3 mo) and 9 O(30 mo)	Eccentric contractions during full electrical stimulation of dorsiflexor muscles, 8x 10 rep, 3/wk during 4,5 wk	–Protein levels of Hsp72, Hsc70 and Hsp25 in muscle –mRNA levels of Hsp72 genes (Hsp70-1, Hsp70-2, Hsp70-3)	–Exercise ↑ Hsp72 and Hsp27 in O, which was 40% lower compared to ↑ in Y –No change in mRNA levels of *hsp72* genes

Table 2. (continued)

References	Population	Type of exercise	Main outcome	Results
Naito et al. (2001)	3 mo (Y) and 23 mo (O) old female Fisher 344 rats, assigned to either a sedentary control or an endurance exercise trained group (N=6 per group)	10 wk running 1 h/d, 5 d/wk on a treadmill at ~77%VO2peak	–Hsp72 in M. Soleus (SOL), M. Plantaris (PL), and the red (RG) and white portions (WG) of the M. Gastrocnemius	–↑ Hsp72 following exercise compared to controls in Y (SOL +22%, PL +94%, RG +44%, WG +243%) and O (SOL +15%, PL +73%, RG +38%, WG +150%) –↑ Hsp72 similar for Y and O in high oxidative muscle, but ↓ in O fast twitch muscle compared to Y
Rinaldi et al. (2006)	6 mo (Y, *N*=6) and 24 mo (O, *N*=18) old male Wistar rats, divided in 3 groups: sedentary young (SY, $n = 6$), sedentary old (SO, n = 8) and trained old (TO, $n = 10$).	6 wk running on treadmill at 30 m/min, 45 min/d 5 d/wk	–Hsp70 and Hsp27 in heart	–↓Hsp70 and ↑Hsp27 in SO compared to SY –↑Hsp70 in TO compared to SO –↑Hsp27 in TO compared to SY and SO
Starnes et al. (2003)	4 mo (Y), 12 mo (A) and 21 mo (O) old male Fischer 344 rats (*N*=60) assigned to control (*N*=10 at each age) or exercise (*N*=10 at each age)	1 h running on treadmill at 70–75% of maximum oxygen consumption	Hsp70 in heart 24 h post exercise	–↑ Hsp70 following exercise: 105% in Y, 27% in A and 24% in O

Table 2. (continued)

References	Population	Type of exercise	Main outcome	Results
Starnes et al. (2005)	40 male Fischer 344 rats, 20 Y (3 mo old) and 20 O (22 mo old), divided in 4 groups (Y and O sedentary, Y & O exercise)	10 weeks on a treadmill 15° incline, 15 m/min for up to 1 h, 5 d/wk	–Hsp70 in heart, liver and M Vastus Medialis –Hsp70-mRNA in heart	–No differences in Hsp70 between sedentary Y & O –Exercise ↑ Hsp70 in muscle of Y (+125%) and O (+70%) –Exercise ↑ Hsp70 in heart (+45%) and liver (+233%) of Y –No change in Hsp70-mRNA in heart
Vasilaki et al. (2002)	6 mo (A, *N*=5) and 28 mo (O, *N*=5) old female Wistar rats	15 min of electrically evoked isometric contractions (0.5s every 5s at 100 Hz/60 V pulse width of 0.1 ms)	Hsp70 & Hsc70 in M. Gastrocnemius 24 h post exercise	–No difference in resting levels between O & A –No change in Hsp70 or Hsc70 in O following exercise –↑ Hsp70 in A following exercise
Vasilaki et al. (2003)	12mo (A) and 30mo (O) old B6XSJL mice	15 min of electrically evoked isometric contractions (0.5 s every 5 s at 100 Hz/60 V pulse width of 0.1 ms)	Hsp25, Hsp25mRNA content and HSF1 binding in M Tibialis Anterior 4, 12 and 24 h post exercise	–No change in Hsp25 and Hsp25mRNA in O, contrary to A (↑) –Similar HSF-1 binding in O & A

Table 2. (continued)

References	Population	Type of exercise	Main outcome	Results
Vasilaki et al. (2006)	12 mo (A) and 30 mo (O) old male B6XSJL mice (*N*=60) allocated to exercise or control	15 min of electrically evoked isometric contractions (0.5 s every 5 s at 100 Hz/60 V pulse width of 0.1 ms)	Hsp70, Hsc70, Hsp25, Hsp25mRNA content and HSF1 binding in M Tibialis Anterior and M.Gastrocnemius immediately, 4, 12 and 24 h post exercise	–No age-difference for Hsp25 in controls –↑Hsc70 in O controls compared to A controls –↑Hsp25 in A following exercise from 4 to 24 h postexercise, no change in O –↑Hsp25mRNA in A immediately following exercise –Hsc70 ↑ in A and ↓ in O 4 h post exercise –↑Hsp70 in A 12 and 24 h post exercise

CR=calorie restriction, Hsp70i=inducible Hsp70, Hsc70=Hsp70 cognate, HSF-1=Heat shock factor-1, d=day, wk=week, mo=month, rep=repetition.

HUMAN STUDIES

Only a limited number of trials in humans were found (N=3), all describing the effect of physical exercise on Hsp70 expression in peripheral mononuclear blood cells (Table 3). Two studies performed by the same group (Simar et al., 2004, 2007) investigated the acute effects of a maximal incremental treadmill test on Hsp72 expression. In the first study, three age-groups were compared: G25 (N=8), G65 (N=12) and G85 (N=8) (mean age 25±1, 66±1 and 82±1 years respectively), all performing regularly physical activity (walking, gymnastics) in an organized setting (Simar et al., 2004). At rest, the percentage of Hsp72-positive lymphocytes was significantly higher in G25 (compared to G85), as well as intracellular Hsp72 expression in monocytes (compared to G65 and G85) and granulocytes (compared to G85). The maximal exercise test induced a significant increase in positive lymphocytes only in G85. Overall, intracellular Hsp72 decreased in granulocytes following the exercise test, which was explained by the authors by possible nuclear translocation of Hsp72 in these cells and post-exercise granulocytosis, thus increasing the proportion of granulocytes less exposed to the exercise stress in the analysis.

The second study compares older subjects (mean age 73±7 years) who were either highly physically active (exercising 10 h/wk at least 9 mo/year, N=16) or were inactive (not engaged in any sports activity, N=16) (Simar et al., 2007). At baseline, the highly active subjects showed a significantly lower number of leukocytes positive for Hsp72 (<50%) and a significantly lower intracellular Hsp72 content (<50%) compared to inactive subjects. Moreover, the highly active group showed higher antioxidant capacity and lower levels of oxidative damage, both significantly related to lower intracellular Hsp72 content. No significant changes in Hsp72 expression were found following the exercise test.

The paper by Bautmans et al. (2005b) was the only study found describing the influence of an intensive strength training program on intracellular Hsp70 expression in older humans. Elderly volunteers (mean age 68±5 years, N=31) performed during 6 weeks 3 times a week intensive resistance exercises (3 series of 10 repetitions at 70– 85% of the maximal strength) for the hip adductors and abductors, knee & hip extensors, shoulder abductors and large trunk muscles. At baseline and following 6 weeks training, resting levels of Hsp70 in monocytes (M) and lymphocytes (L) were determined both without and after application of a heat shock (incubation at 42°C). The amount of Hsp70 expression in M and L determined without heat shock decreased significantly after the training program. In contrast, heat shocked M and L (at 42°C) produced significantly higher amounts of Hsp70 at the end of the six-week strength training compared to baseline.

Both the crossectional study by Simar et al. (2007) and the prospective intervention study by Bautmans et al. (2005b) describe an attenuation of basal Hsp70 expression in PBMC of elderly persons following regular physical exercise, which is in agreement with findings in young human subjects (Fehrenbach et al., 2000a, b; Shastry et al., 2002). Possibly, this phenomenon reflects immunologic adaptations. In fact, it is assumed that regular physical exercise has a strong regulating effect

Table 3. Studies describing the influence of physical exercise on HSP-expression in elderly humans

References	Population	Type of exercise	Outcome	Results
Bautmans et al. (2005b)	10 male, 21 female, age 68±5 years	6 weeks IST	Hsp70 in PBMC – (IAS) – After heat shock (42°C)	– ↓ Hsp70 IAS after IST – ↑ Hsp70 42°C after IST
Simar et al. (2004)	3 age groups: – G25: age 25±1 years, 4 male, 4 female – G65: age 66±1 years, 8 male, 4 female – G85: age 82±1 years, 3 male, 5 female	MITT	– PBMC positive for Hsp72 – Hsp72 in PBMC	– ↑ Hsp72 positive Leukocytes in G85 after MITT – ↓ Hsp72 in granulocytes in all groups after MITT
Simar et al. (2007)	– 16 highly active (8 male and 8 female) – 16 low active (8 male and 8 female) – age 73±7 years	MITT	– Leukocytes positive for Hsp72 – Hsp72 in PBMC	– >50% lower Hsp72 in PBMC of highly active compared to low active subjects – no influence of MITT

PBMC=Peripheral blood mononuclear cells, IST= intensive strength training, IAS= immediately after sampling, MITT= maximal incremental treadmill test.

on systemic inflammatory processes such as seen in cardiovascular disease and the basal low-grade inflammatory profile in elderly (Pedersen and Bruunsgaard, 2003; Petersen and Pedersen, 2005). In this context, the exercise-induced bouts of IL-6 elevations in the circulation may play an important signaling function. The exercise schedules as described in the study of Bautmans et al. (2005b) were sufficiently intensive to elicit significant increases of IL-6 serum levels. As well, they observed a trend of resting IL-6 levels to decrease after the 6 weeks training program. Thus, the lower basal intra-cellular Hsp70 expression in trained elderly persons might reflect less systemic inflammatory activity. In fact, the exposure to (repetitive) mild stress has been shown to improve survival and longevity both at the cellular and organism level and Hsp-expression might play an important role (for review Minois, 2000; Horowitz and Robinson, 2007).

The optimal schedule (type of exercise, intensity, duration, frequency) for physical exercise in the elderly remains speculative. In fact, also in the elderly there exists a strong dose-response relationship following exercise (Williams et al., 2007). When targeting the age-related muscle weakness, it is now clear that intensive resistance exercise is the most efficient modality. Based on the studies in young subjects, it appears that the HSP-response is dependent on exercise intensity, as can be demonstrated both at the level of the muscle itself (Liu et al., 2000) as in PBMC (Shastry et al., 2002; Whitham et al., 2004). Resistance training resulted in larger increases in intramuscular Hsp70 compared to endurance training (Liu et al., 2004). Possibly, the lack of acute increases in intracellular Hsp70 expression following the maximal exercise test in the studies by Simar et al. (2004, 2007) might, besides a too low power given the low sample size, have been due to a combination of an age-related decrease in the capacity to respond to physical stress and a too low intensity of the treadmill exercise. In analogy, Shastry et al. (2002) also conclude, after comparing HSP-responses following physical exercise in young persons, that "exercise at an intensity that is within normal limits for moderately trained individuals is not a sufficient stimulus of Hsp70 production in leukocytes". Thus, it seems that more intensive exercises are necessary to elicit acute HSP-responses in PBMC and muscle cells and that intensive resistance exercises might be a preferential choice in the aged. It has to be noted that, when prescribing intensive training in older persons, it is important to increase the exercise intensity progressively and to introduce a sufficient recovery period between the training sessions, in order to allow recovery from the exercise-induced oxidative stress. In fact, it is well known that intensive muscle contractions produce oxidative stress in the tissues (Coffey and Hawley, 2007), one of the mechanisms triggering the HSP-response. Due to an accumulation of non-enzymatic cross-links (AGE) in the aged muscles and their interaction with RAGE, the oxidative stress is already increased in the tissues, thus potentially impairing the resistance against supplementary exercise-induced oxidative stress. Too abrupt and unbalanced training programs in untrained elderly, who show impaired stress-induced cellular defense mechanisms (HSP-response), might lead (initially) to increased cell damage and higher risks for injury. Given the higher accumulation of AGE's in diabetic persons, elderly persons with diabetes mellitus and insufficiently regulated

hyperglycemia might therefore be at higher risk (as recently suggested in animal models (Stoppa et al., 2006)).

The improvement of heat-induced Hsp70 expression in PBMC as seen following 6 weeks resistance training in the study of Bautmans et al. (2005b) has also been described in young trained athletes (Fehrenbach et al., 2000a) and might reflect a better cellular protection during stressful situations. In this context, although not investigated yet, it can be hypothesized that training-related adaptations of intracellular Hsp70 expression might play a role in the improved wound healing by physical training, as recently described in old mice (Keylock et al., 2008) and in elderly humans (Emery et al., 2005).

CONCLUSION

In summary, ageing is related to significant loss of muscle mass and muscle strength, called sarcopenia. The underlying mechanisms are closely related to inflammatory processes and it is assumed that these are aggravated by concomitant age-related alterations in cell-protecting mechanisms involving HSP, especially the Hsp70-family. The most effective modality to counter sarcopenia is intensive resistance exercise. Physical exercise induces, in a dose-response relationship, an acute phase reaction and influences Hsp70 expression in PBMC and several tissues. In this review we focused on the influence of physical exercise on the HSP expression during aging. Following a systematic literature search it can be concluded that there is very limited information available at this moment. Animal studies describe a blunted exercise-induced HSP response in the skeletal muscle, heart and liver of older rodents compared to younger ones. The exact origin of this age-related reduction is not yet identified. Based on the studies that were included in our review it seems that this phenomenon is not caused by reduced HSF1 availability or capacity to make mRNA in response to exercise. Only three reports involving elderly human subjects were identified in the literature search, from which two crossectional and one prospective intervention study. From these studies we can conclude that physical exercise lowers the basal Hsp70 expression in human PBMC, probably by reducing the low-grade inflammation and the oxidative stress. A 6-weeks intensive resistance training program at higher age improves the heat-induced Hsp70 expression in PBMC, a phenomenon which might be related to a better cellular protection during stressful situations and an accelerated recovery process following injury or disease in trained versus untrained elderly persons. Comparisons between exercise-induced HSP-responses in elderly animal and human subjects are difficult to perform given the different substrates used in these experiments (tissues of muscle, heart, liver in animals versus PBMC in humans). As a final conclusion it appears that physical exercise in elderly can have an important regulating effect on inflammatory processes and cellular protection mechanisms involving HSP-expression. Since this area has been incompletely studied at this moment, more research is warranted, especially

involving elderly persons in different clinical conditions and exploring the effects of different exercise schedules.

APPENDIX

The bibliographic databases PubMed and Web of Science were screened (last search performed on August 7th 2008) systematically for *experimental trials* targeting the HSP-response to physical exercise in the elderly. For the search the following search keys were used:

PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez/query.fcgi):

(Aged OR Aged, 80 and over OR Ageing OR Aging OR Elderly) AND (Exercise OR Exercise Therapy OR Exercise Movement Techniques) AND (Heat-Shock Proteins OR Heat-Shock Response OR Chaperones OR Stress proteins) Keywords in this search were used both as Medical Subject Heading (MeSH, the U.S. National Library of Medicine controlled vocabulary thesaurus used for indexing MEDLINE articles) and as free keywords (as text).

Total hits=183, from which relevant trials N=15 (3 on humans, 12 on animals)

Web of Science (http://apps.isiknowledge.com):

(Aged OR Ageing OR Aging OR Elderly) AND (Exercise OR Exercise Therapy OR Exercise Movement Techniques) AND (Heat-Shock Proteins OR Heat-Shock Response OR Chaperones OR Stress proteins)

Total hits=92, from which relevant trials N=12 (3 on humans, 9 on animals)

Papers were selected when they corresponded to all of the following criteria:

– full article, reporting a trial (reviews and conference abstracts were excluded),
– article involving older humans (age≥60 years) or older animals
– investigating the influence of physical exercise on HSP-expression

Fifteen trials corresponded to these criteria, from which three on humans and 12 on animals.

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CHAPTER 22

HEAT SHOCK PROTEINS, EXERCISE, AND AGING

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Abstract Age-associated losses in muscle mass and strength and greater susceptibility to muscle injury may be attenuated or reversed by exercise training. Increased heat shock protein (HSP) expression is one adaptation in skeletal muscle to exercise which may subsequently protect muscles against stressors. Eccentric loading, treadmill running, resistance training, and functional overload have been shown to increase Hsp72 and Hsp25 content in muscle. Evidence suggests that the muscles of older individuals can respond to exercise with increased HSP expression; however, the responses may be attenuated compared to younger muscles. A sufficient exercise stimulus may be necessary to induce up-regulation of protective HSP in muscle such that even modest reductions in muscle activity and loading that occur with aging may contribute to reduced basal or stress-induced HSP responses in aged muscle. This could contribute to some of the deleterious changes in muscle function since HSP potentially protect muscles against contractile damage, accelerate recovery from injury and reduce activation of inflammatory pathways such as NF-κB and the caspase cascade. The prospect that exercise may increase expression of HSP in aged muscle could have important implications for increasing muscle strength and function and improving quality of life

Keywords Age-related sarcopenia; exercise; HSP; aging; NF-κβ

Abbreviations: HSF1, heat shock factor 1; HSP, heat shock protein; MHC, myosin heavy chain; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells;

INTRODUCTION

Skeletal muscle comprises the largest organ system in the body and is indispensible for the activities of daily living as well as exercise; however, aging is associated with a significant loss of muscle mass and strength a condition known as sarcopenia

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(Karakelides and Sreekumaran Nair, 2005). Many factors may contribute to sarcopenia including inactivity, inflammation, free radical damage, and apoptosis. Exercise is an intervention that reliably counteracts the decline in muscle mass, strength and power that accompanies aging (McArdle and Jackson, 2000). Moreover, with age, skeletal muscles become more susceptible to damage and have a diminished ability to regenerate successfully after damage (Broome et al., 2006; Kayani et al., 2008). This exacerbates age-related functional declines leading to loss of mobility and increased risk of mortality (Degens, 2007). Although the mechanisms behind these age-related deficits in skeletal muscle function are unclear, reduced physical activity combined with an attenuation of the ability of skeletal muscles to express protective proteins in response to physical stresses likely play major roles. In particular, increased expression of heat shock proteins (HSP) is one of the many important acute and/or chronic adaptive responses in skeletal muscle to exercise which may subsequently protect muscles against stressors such as mechanical damage or oxidative stress (Locke and Noble, 1995; Nishimura and Sharp, 2005). The goal of this chapter is to summarize the current knowledge regarding the major effects of aging on the HSP response to exercise with particular focus on skeletal muscle. This goal will be accomplished by providing a summary of HSP responses – primarily Hsp72 and Hsp25 – to exercise in younger as compared to older individuals and by providing evidence of potential mechanisms whereby HSP may protect muscles against damage and loss of strength and function as one ages.

The family of HSP are considered an endogenous defense system that enables cells to survive stressful situations such as oxidative stress and inflammation (Latchman, 2001; Locke and Noble, 1995; Moseley, 1998; Welch, 1992). The family of HSP proteins includes large (Hsp60, Hsp70, Hsp90 and Hsp100) and small (Hsp10, Hsp25 (human homologue is Hsp27) αβ-crystallin, and Hsp40) proteins. The Hsp70 family is known for its chaperone properties (Macario and Conway de Macario, 2005; Morimoto, 1998), is highly expressed in skeletal muscle and is the most well-characterized HSP family in skeletal muscle adaptation and disease (Liu et al., 2006; Liu and Steinacker, 2001; Locke and Noble, 1995; Nishimura and Sharp, 2005). Within the Hsp70 family, Hsp72 is highly inducible and can help re-fold proteins as well as chaperone damaged proteins to the ubiquitin-proteosome system for subsequent degradation (Georgopoulos and Welch, 1993). Note: In some studies, Hsp70 is used to refer to the inducible form of Hsp70 (i.e. Hsp72). Hsp25 and αβ-crystallin are abundantly expressed in muscle, particularly slow oxidative muscle (Bhat and Nagineni, 1989; Golenhofen et al., 2004; Huey et al., 2004; Inaguma et al., 1993), are structurally and functionally related (Merck et al., 1993), and are highly inducible in response to numerous stressors. Importantly, evidence suggests that the inherent stress of exercise may increase HSP expression in active muscles and consequently provide protection against future damaging stressors such as inflammation or muscle damage.

Interventions to help preserve or increase muscle mass and strength as an individual ages have included aerobic exercise, resistance exercise, and/or nutritional supplementation. While the benefits of exercise are widely acknowledged and

include increased muscle strength and endurance in both young and old individuals, the responses of HSP during muscular adaptation to exercise are incompletely understood, especially in aged individuals. Importantly, muscular exercise is associated with up-regulation of HSP expression in active skeletal muscles in younger individuals; however, limited evidence suggests that this response may be attenuated in older muscles and thus may contribute to reductions in muscle function with aging. As evidence, transgenic over-expression of HSP70 in the mouse extensor digitorum longus muscle was associated with maintenance of specific tension and improved recovery from contractile activity in older mice (McArdle et al., 2004). Thus, one hypothesis is that exercise training, which has been shown to increase HSP levels in adult skeletal muscles, could help preserve muscle function as one age in part through elevated HSP levels.

EFFECTS OF MUSCLE LOADING AND ACTIVITY (I.E. EXERCISE) ON HSP EXPRESSION

While the skeletal muscle HSP response to exercise has been the focus of numerous studies, only recently has subject age been taken into account. The existing in vivo data suggests that increases in muscle activity and loading are associated with increased expression of both large and small HSP in the active muscles of adult humans and rodents. Several different models of increased muscle loading and activation have been used both acutely and chronically to increase HSP content in muscle and include eccentric loading (Feasson et al., 2002; Koh and Escobedo, 2004; Paulsen et al., 2007; Thompson et al., 2001), treadmill running (Ecochard et al., 2004; Kayani et al., 2008; Locke et al., 1990; Morton et al., 2006; Puntschart et al., 1996; Salo et al., 1991; Skidmore et al., 1995), resistance training (Gjovaag and Dahl, 2006; Murlasits et al., 2006), and functional muscle overload (Huey, 2006; Huey et al., 2007; O'Neill et al., 2006; Ogata et al., 2005; Oishi et al., 2005). A single bout of eccentric loading, which is also a strong stimulus for muscle injury, is associated with significant increases in Hsp70 and Hsp25 in both humans and rodents. In humans, one bout of novel eccentric contractions increased Hsp72 and Hsp25 protein levels ~10- and 2-fold, respectively, in the biceps brachii 48 h after exercise (Thompson et al., 2001). Another model of eccentric exercise, 30 min of downhill running, was associated with increases in Hsp27 and αβ-crystallin in human quadriceps 24 h after exercise (Feasson et al., 2002). Unaccustomed eccentric exercise not only alters HSP expression, but also cellular localization. For example, unaccustomed eccentric exercise of the human quadriceps was associated with 15- and 8-fold increases in Hsp27 in the myofibrillar fraction 0.5 or 4 days, respectively, after exercise (Paulsen et al., 2007). After 4 days, 2- and 10-fold increases in Hsp70 were observed in the cytosolic and myofibrillar fractions, respectively (Paulsen et al., 2007). An immediate translocation of Hsp25 to the myofibrillar (insoluble) fraction has also been shown in the rat extensor digitorum longus after lengthening contractions (Koh and Escobedo,

2004). In both humans and rodents, this translocation of HSP to the myofibrillar fraction has been suggested to potentially limit cytoskeletal disruption and/or assist in repairing denatured or damaged muscle proteins (Koh, 2002; Koh and Escobedo, 2004; Paulsen et al., 2007). While acute damaging exercise increases both large and small HSP, a single bout of non-damaging treadmill exercise has been associated with increases in Hsp72 in both humans and rodents (Locke et al., 1990; Morton et al., 2006; Puntschart et al., 1996; Salo et al., 1991; Skidmore et al., 1995). While, the study by Morton and colleagues was the only investigation to characterize the response of the small HSP, Hsp27 and αβ-crystallin, their results suggest that muscle damage may be important for increases in Hsp27 and αβ-crystallin as only Hsp72 was increased in human skeletal muscle following a bout of non-damaging exercise (Morton et al., 2006).

While HSP are responsive to single bouts of exercise – especially damaging exercise – chronic exercise training also impacts the HSP response in active skeletal muscles. For example, our lab has shown that in a rodent model of functional muscle overload the chronic increases in muscle loading and activation over 7 days were associated with time-dependent increases in Hsp25 expression and phosphorylation in both slow and fast hindlimb muscles (Huey, 2006; Huey et al., 2007). However, these responses were differentially impacted by the myosin heavy chain (MHC) isoform profile of the muscle (i.e. muscle fiber type). While under basal conditions, Hsp25 is more highly expressed in slow type I fibers (Golenhofen et al., 2004; Huey et al., 2002; Inaguma et al., 1993), increased muscle activity may be critical in increasing Hsp25 in all muscle fiber types as evidenced by the greater relative increases in Hsp25 in the plantaris, which is composed predominately of fast type II MHC fibers as compared to the soleus which is composed primarily of slow type I MHC fibers (Huey, 2006; Huey et al., 2007). Increases in Hsp72 in functionally overloaded rodent muscle has also been shown and some evidence suggests that increases in Hsp72 in the fast plantaris may be related to the shift to a slower MHC profile with chronic overload (O'Neill et al., 2006; Ogata et al., 2005; Oishi et al., 2005). This muscle fiber type specific response could have important implications in aged muscle as it has been shown that aging is associated with a greater loss of fast type II fibers as compared to slow type I fibers (Degens, 2007). While functional overload in rodents forces the muscles to adapt to higher sustained loads, chronic muscle activity independent of high loading also increases Hsp72. Specifically, treadmill training is associated with elevated levels of Hsp72 in active rodent skeletal muscles (Ecochard et al., 2004; Kayani et al., 2008). Humans trained with rowing exercise also have elevated Hsp72 levels in active skeletal muscles (Liu et al., 1999).

In humans, increases in Hsp72 and Hsp25 after 5–8 weeks of elbow extensor exercise were independent of both exercise intensity and duration (Gjovaag and Dahl, 2006). Exercise training was found to increase Hsp72 and Hsp25 protein levels 111 and 71%, respectively; however, the increases in HSP expression were inversely related to the basal level of expression (i.e. individuals with low pre-training values showed the greatest HSP response) (Gjovaag and Dahl, 2006). Our lab has also shown that in response to chronic muscle overload, the soleus – a slow

oxidative muscle with high baseline Hsp25 expression – exhibited an attenuated relative Hsp25 response compared to the plantaris, a fast glycolytic muscle with relatively lower baseline HSP expression (Huey, 2006; Huey et al., 2007). This has also been shown at the transcriptional level as individuals with the lowest basal levels of Heat Shock Factor-1 (HSF-1) DNA binding had the highest responses following exercise (Palomero et al., 2008). Taken together, this suggests that once a threshold level of HSP protection is reached, greater stimuli maybe necessary for additional increases in HSP expression. On the applied level, this may imply that older individuals who exercise regularly may have to increase exercise intensity or duration to elicit further increases in HSP expression in skeletal muscle (discussed below).

EFFECTS OF MUSCLE LOADING AND ACTIVITY ON HSP EXPRESSION: EFFECTS OF AGING

While in younger individuals, the existing literature suggests that HSP respond to an exercise stimulus with up-regulation and/or translocation, the responses in aged individuals are conflicting; most probably due to the limited number of studies. The studies have suggested, however, that the inability to produce adequate HSP in response to a physical stimulus may play an important role in age-related declines in muscle function.

For example, Murlasits and colleagues found that while resistance training increased the expression of Hsp72 and Hsp25 in the muscles of both young (3 months) and older (30 months) rats, a greater response was observed in the young mice (Murlasits et al., 2006). Specifically, for both Hsp72 and Hsp25 the protein responses to resistance training in young muscles were approximately double the responses observed in older muscle. Further, in a small number of older animals, the data suggested that the Hsp72 response in aged muscle was attenuated by the higher levels of oxidative stress in aged muscles (Murlasits et al., 2006). In contrast, stretch-induced hypertrophy of slow quail muscles was not associated with any increases in Hsp72 or Hsp25 in the muscles of old animals, despite significant increases in young muscles after 7 days (Siu and Alway, 2005). Similarly, Hsp25 protein levels were significantly increased in adult mouse muscle 4, 12, and 24 h after a single bout of isometric exercise, but no changes were observed in old mouse muscle (Vasilaki et al., 2006). In another study utilizing the same isometric exercise protocol, significant increases in Hsp70 protein in adult, but not aged muscle was found 24 h after exercise (Vasilaki et al., 2002). The lack of a significant up-regulation of HSP expression in aged muscle may contribute to the differences in muscle adaptation to chronic stretch-shortening training for 4.5 weeks (Cutlip et al., 2006). Adult muscle responded to this training program with muscle hypertrophy and improved performance while aged muscle had a significant performance decline and indications of inflammation (Cutlip et al., 2006). Thus, since HSP may help preserve muscle integrity and assist in muscle repair, a reduced HSP response in aging muscle

may be one factor contributing to the failure of aged muscle to adapt rigorously in the face of overload.

The effects of age on the skeletal muscle HSP response has also been studied in response to treadmill training. Following 10 weeks of treadmill exercise, increases in Hsp72 were found in both young and old rats; however, the responses were muscle fiber type specific (Naito et al., 2001). Aging was not associated with a reduced Hsp72 response in oxidative muscles expressing primarily slow type I MHC (soleus, red gastrocnemius), but the response was blunted in glycolytic muscles expressing primarily faster type II MHC (plantaris, white gastrocnemius). Kayani et al reported that 10 weeks of treadmill training increased Hsp70 expression in the muscles of adult mice, but not aged mice (Kayani et al., 2008). Further, mice trained for 12 months (starting at 12–14 months old) also did not show any increases in skeletal muscle Hsp70 (Kayani et al., 2008). However, a modest training program of only 15 min of running per day (15 m/min, 0 degree grade), 3 days a week was utilized which may not have been a sufficient stimulus to increase HSP content. The absence of an increase in Hsp70 in active muscles may have contributed to the finding that training did not improve the recovery of aged muscles from damaging contraction (Kayani et al., 2008) as had been previously shown when Hsp70 was over-expressed in aged mouse muscle (Broome et al., 2006). A study by Starnes et al. demonstrated that greater exercise intensity and duration is associated with increases in skeletal muscle Hsp70 in both adult (6 month old) and aged (22 month old) rats (Starnes et al., 2005). In this study, rats ran 5 days/wk at 15 m/min, 15 degree grade for 60 min/day and Hsp70 levels in the vastus medialis were increased 125 and 70%, respectively, in young and older rats relative to sedentary controls. Interestingly, in the aged animals, skeletal muscle was the only tissue studied to show training-induced increases in Hsp70 as exercise training failed to increased Hsp70 expression in heart or liver tissue despite significant increases in the trained young animals relative to sedentary controls (Starnes et al., 2005). Taken together, these findings suggest that age-related changes in HSP70 expression may depend upon the level of stress and may be tissue specific. While no studies have quantified Hsp72 expression in the skeletal muscles of humans following endurance training, one group did report that long term exercise training (12 months) was associated with significant decrease in Hsp72 content in leukocytes (Simar et al., 2007). Those authors suggested that this down-regulation may have been the result of lower levels of oxidative damage attributable to up-regulation of endogenous anti-oxidant defenses with exercise training (Simar et al., 2007).

The existing data in skeletal muscle is consistent in showing that age can have significant effects on the muscle's ability to up-regulate HSP expression in response to either acute or chronic exercise. This is further supported by data in another type of striated muscle – cardiac muscle – in which reduced HSP responses have also been associated with aging. For example, while Hsp72 expression in the myocardium was increased in both young (6 month) and older (24 month) Fisher 344 rats following either heat stress or treadmill training, the responses were attenuated in aged when compared to adult myocardium (Demirel et al., 2003). Further, these investigators

found that heat stress was also associated with significant increases in myocardial Hsp72 in both young and older heat-stressed rats compared to controls; however the responses were again attenuated in the older rats and the responses in both heat-treated groups were lower than those observed in response to exercise training. Several investigators have also reported an attenuated up-regulation of rat myocardial Hsp70 in response to acute thermal stress in aged when compared to young (Kregel et al., 1995; Locke and Tanguay, 1996). While all these studies reported similar Hsp70 protein levels in the myocardium of younger and older rats (Demirel et al., 2003; Kregel et al., 1995; Locke and Tanguay, 1996), a different study reported that Hsp70 levels were significantly lower in sedentary aged (24 months) as compared to sedentary young (6 months) Wistar rats. In this study, the aged rats were exercise trained for 6 weeks to determine if Hsp70 expression in the myocardium could be increased to the Hsp70 levels observed in young sedentary rats. Six weeks of treadmill training did significantly increase Hsp70 protein expression in older rat myocardium; however, Hsp70 levels in aged rat myocardium still failed to reach the levels observed in young sedentary rat myocardium (Rinaldi et al., 2006). One potential difference between this study (Rinaldi et al., 2006) and the earlier studies (Demirel et al., 2003; Kregel et al., 1995; Locke and Tanguay, 1996), which found no age-related differences in baseline myocardial Hsp72 expression, was the strain of rat as the earlier studies utilized Fisher 344 rats and Rinaldi et al. used Wistar rats. Fisher 344 rats are a well established model for the study of aging as senescence occurs early at 24 months and this strain of rat has been well-characterized under defined environmental and genetic conditions regarding age-associated changes whereas Wistar rats have not.

The evidence that exercise training is associated with a greater up-regulation of Hsp70 in the aged rat myocardium as compared to thermal stress may have important clinical implications given the reduced cardiac function that accompanies aging. Changes occur in the cardiac myocyte with age at the macroscopic as well as the cellular and molecular levels (Bernhard and Laufer, 2008). Moreover, these changes are initiated by intrinsic oxidative stress and damage as well as extrinsic elements, such as lifestyle factors. Consequently, exercise may be one way to enhance cellular protective responses in the cardiac myocyte thereby providing some protection against age-related cardiac dysfunction.

Taken together, increases in Hsp72 and/or Hsp25 with exercise training are consistent across human and animal studies especially in younger subjects, but the increases have been shown to be maintained only if the training stimulus is held constant or increased (Gjovaag and Dahl, 2006; Liu et al., 2000, 2004). Just as the reductions in muscle activity and loading that occur with detraining may reduce HSP content in skeletal muscle, profound muscle disuse is clearly associated with decreased HSP expression. Our lab has shown that within a week of disuse, Hsp25 is significantly decreased (Huey et al., 2004) and this reduction in Hsp25 content is maintained for up to 30 days of disuse (Huey et al., 2005). We have also found that during 28 days of muscle atrophy induced by spinal cord injury, time-dependent reductions in muscle mass were significantly correlated with the loss of Hsp25 in

several rat hindlimb muscles (Huey et al., 2008). Hsp72 has also been shown to be significantly reduced in skeletal muscles subjected to chronic disuse. Oishi et al. reported that 5 days of hindlimb unloading combined with denervation or tenotomy was associated with significant decreases in Hsp72 in the rat soleus and plantaris (Oishi et al., 2001). Eight days of hindlimb unloading was also associated with significant reductions in Hsp72 in the rat soleus while prior heat stress increased Hsp72 content and this was associated with reduced muscle atrophy (Naito et al., 2000). Further, heat stress prior to lower hindlimb immobilization increased Hsp25 and Hsp72 in rat soleus and this was associated with reduced atrophy following 8 days of immobilization (Selsby and Dodd, 2005)

Looking at the continuum from exercise training to detraining to muscle disuse, it is evident that muscle loading and activation can stimulate skeletal muscle HSP expression under certain conditions. However, a comparison of the studies by Kayani et al. (2008) and Starnes et al. (2005) highlights that a sufficient exercise stimulus may be necessary to induce up-regulation of protective HSP in skeletal muscle. Further, it is possible that even modest reductions in muscle activity and loading that commonly occur with aging may contribute to reduced basal or stress-induced HSP responses in aged skeletal muscle and subsequently contribute to some of the deleterious changes in muscle function that occur with aging.

MECHANISMS FOR THE PROTECTIVE EFFECTS OF HSP IN AGING MUSCLE NUCLEAR FACTOR- κB (NF- κB)

Aging is associated with elevated inflammatory markers both systemically (Muller-Werdan, 2007) and specifically in skeletal muscle (Meador et al., 2008) in response to an acute inflammatory insult. Both Hsp25 and Hsp70 have been shown to reduce NF-κB activation (Carlson et al., 2007; Chen et al., 2005; Kammanadiminti and Chadee, 2006; Meldrum et al., 2003; Park et al., 2003; Yenari et al., 2005), which is central to the expression of several pro-inflammatory cytokines (Karin, 1998; Viatour et al., 2005). While exercise has been shown to acutely activate NF-κB (Ho et al., 2005; Ji et al., 2004), chronic activation of this pathway has been associated with muscle weakness (Cai et al., 2004) and contractile protein loss (Ladner et al., 2003; Li and Reid, 2000). Further, greater activation of NF-κB during muscle immobilization has been reported to occur in aged as compared to younger rat skeletal muscle (Bar-Shai et al., 2005) which can be critical as the potential for injury or illness leading to sustained inactivity greatly increases with age. In non-muscle cells, Hsp70 and Hsp25 have been shown to reduce inflammation-mediated NF-κB activation by decreasing inhibitor of IκB kinase (IKK) activity (Chen et al., 2005; Kammanadiminti and Chadee, 2006; Park et al., 2003; Yenari et al., 2005). In vivo data suggesting interactions between HSP and the pathways activating NF-κB were reported using prior heat stress in a model of angiotensin II induced hypertension. Significant increases in Hsp25 and Hsp70 expression in rat aorta were temporally associated with significant reductions NF-κB activation (Chen et al., 2004a, b). Recent work in obese mice

has also shown that increased expression of Hsp72 in skeletal muscle was associated with reduced c-jun amino terminal kinase (JNK) phosphorylation in skeletal muscle and reduced IKK phosphorylation in the liver (Chung et al., 2008). Therefore, existing evidence suggests that several HSP may attenuate transcriptional activation of NF-κB thereby potentially reducing the expression of pro-inflammatory cytokines and the negative consequences they may have on muscle strength and function.

Caspases

While apoptosis is critical in maintaining tissue integrity, especially in proliferative tissues, up-regulation of apoptotic pathways in skeletal muscle constitutes a major mechanism by which aged muscle mass declines (Marzetti and Leeuwenburgh, 2006). For example, Siu and colleagues reported that aging was associated with greater activation of pro-apoptotic signaling during hindlimb unloading as compared to adult rats (Siu et al., 2005). Caspases are involved in both the initiation (caspase -8, -9, -12) and execution of apoptosis (caspase -3, -6, -7). Activation of caspase-3, a cysteine dependent protease, is likely a critical rate-limiting step for myofilament release and is strongly implicated in accelerated contractile protein breakdown (Du et al., 2004; McClung et al., 2007; Rachek et al., 2007) and muscle weakness.

Interestingly, both large and small HSP may inhibit activation of caspase-3 and attenuate the devastating consequences of sarcopenia. Hsp25 or αβ-crystallin overexpression in non-muscle tissues has been shown to significantly reduce caspase-3 activity in vivo (Akbar et al., 2003; Lee et al., 2006) and in vitro (Choi et al., 2007; Morrison et al., 2003; Pandey et al., 2000) likely by inhibiting cleavage of pro-caspase-3 to active caspase-3 (Kamradt et al., 2001; Pandey et al., 2000). While direct Hsp25 or αβ-crystallin inhibition of caspase-3 activity in skeletal muscle, in vivo, has not been shown, our recent publication reported that the loss of Hsp25 was significantly correlated with increased caspase-3 activity in atrophying rat plantaris (Huey et al., 2008). Several studies have shown that Hsp72 blocks stress-induced activation of the caspase cascade (Yenari et al., 2005), but also has the ability to function upstream of mitochondria and caspase activation (Gabai et al., 2002). In summary, since caspases are important initiators of muscle breakdown, increased expression of HSP in skeletal muscle can potentially help preserve muscle integrity and strength especially in response to stressors known to activate the caspase cascade such as inflammation and injury.

CONCLUSION

The protective effects of the family of HSP in skeletal muscle are highly relevant to the aging population because of the high incidence of sarcopenia and the devastating effect muscle loss has on health and mobility. The potential for exercise to

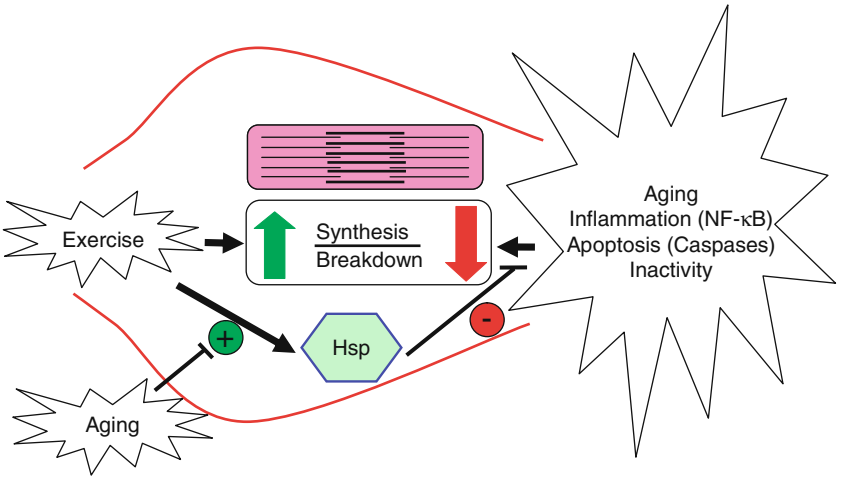

Figure 1. Proposed model for the effects of aging and exercise on HSP expression in muscle and subsequent effects on inflammatory and apoptotic pathways that alter the ratio of muscle protein synthesis and degradation. Exercise has been shown to increase HSP expression in active muscles; while, limited evidence suggests that aging may attenuate these increases. However, higher exercise intensities or durations may be effective in increasing HSP expression in aged muscles which could subsequently protect muscles from the myofibrillar breakdown associated with NF-κB or caspase activation.

increase expression and/or phosphorylation of HSP in aged muscle may have important implications for increasing muscle strength and function and improving quality of life.

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CHAPTER 23

HSP60 AND HSP10 IN AGEING

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Abstract: HSP and molecular chaperones, both referred to in this chapter as chaperones, are key players in development and senescence. With regard to senescence, several issues are critical: the role of normal chaperones in the process of ageing itself and in preventing and controlling age-associated diseases, the role of defective chaperones (chaperonopathies) in the onset and progression of senescence and in the etiology of old-age diseases, the interaction of chaperones with the immune system, and the potential of chaperones as therapeutic agents for counteracting the deleterious effects of ageing on molecules and cells and for treating proteinopathies of the elderly (chaperonotherapy). All these issues are discussed in this chapter, focusing on Hsp60 and Hsp10 (referred to as chaperonins for the purposes of this article) by examining a sample of publications dealing with pathological conditions prevalent in aged individuals, such as atherosclerosis and coronary syndromes, neurodegenerative and degenerative joint disorders, diabetes, chronic occlusive pulmonary disease, and glaucoma (cancer is omitted because it has recently been extensively discussed elsewhere). The data show that Hsp60 and Hsp10 undergo changes in levels and distribution inside cells and tissues, including invasion of the extracellular space and plasma, with age and health status and in relation with specific pathologies. The physiological and/or pathological significance of these changes is not yet fully understood but is being actively investigated. The role of chaperonopathies, particularly those due to aberrant post-translational modifications related to stressors such as ROS, on the aggravation of senescence ought to be examined in detail in the near future. Studies on the mechanisms by which defective chaperones (molecular chaperonopathies) accelerate senescence and contribute to pathogenesis and, thus, to the development of disease (clinical chaperonopathies) should provide key information useful for developing diagnostic and therapeutic means based on chaperone genes and proteins

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Abbreviations: AD, Alzheimer's disease; ATS, atherosclerosis; CAD, coronary artery disease; CNS, central nervous system; CP, *Clamydia pneumoniae*; DAVS, degenerative aortic-valve stenosis; ECC, extracorporeal circulation; ERK, extracellular signal-regulated kinases; her2, human epidermal growth factor receptor2; HSP, heat shock proteins; IL-6, interleukin-6; LDL, low density lipoproteins; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PD, Parkinson's disease; Th-1, T helper-1; TLR, toll-like receptors; TNF-α, tumor necrosis factor-alpha

INTRODUCTION

Chaperonology, the field of science encompassing the study of HSP and molecular chaperones (henceforth referred to as chaperones) is emerging as a new area of physiology and molecular biology with significant impact on other fields, such as genomics, pathology, and medicine (Macario and Conway de Macario, 2007a). Defective chaperones can contribute to pathogenetic mechanisms in pathologic conditions now known as chaperonopathies (Macario, 1995; Macario and Conway de Macario, 2002, 2004). Definition of these conditions as belonging to a specialized subfield of pathology and their classification according to their origin is one of the landmarks in the study of chaperones since they were discovered in 1962 (Haak and Kregel, 2008). Chaperonopathies can be genetic or acquired and, particularly the latter, are quite common (Macario et al., 2005; Macario and Conway de Macario, 2005). Identification of genetic chaperonopathies and, consequently, the realization that polymorphisms and defects (mutations and/or dysregulation) in genes encoding chaperones, have lead to the development of chaperonomics (Brocchieri et al., 2006), which chiefly aims at identifying the total complement of genes in any given genome that encode chaperones and other, functionally related, molecules such as nucleotide exchange factors and tetratricopeptide repeat-containing proteins that interact with chaperones (Soti and Csermely, 2007a).

Fundamental issues pertaining to chaperones and ageing include the changing roles of these proteins in cell physiology from embryo through old age, particularly their roles in the mechanism of cellular senescence, and the impact of defective chaperones might have on senescence at all levels, from intracellular to organismal processes, including cellular and tissue alterations characteristic of senescence. Does the age-associated appearance of defective chaperones (chaperonopathies of ageing) due to molecular damage precede the appearance and accumulation of defective non-chaperone proteins (proteinopathies of ageing) and allow this accumulation to begin and to generalize to include many different proteins, and to progress unchecked? If that were the case, one might say that chaperonopathies are the primary pathogenetic mechanism of multiple aspects of senescence. Chaperone failure would cause the

initiation and progression of proteinopathies, and also of other pathological manifestations affecting cellular processes in which chaperones take part. Alternatively, do the chaperonopathies and the proteinopathies of ageing start independently of one another, perhaps simultaneously, and progress in parallel with the years, with the chaperonopathies having a negative impact on protein homeostasis and thus contributing to the aggravation of the proteinopathies? In this situation, the chaperonopathies would be a contributing factor to senescence, an important factor but not the primary one. A third possibility would be that chaperonopathies do not play any significant role in the ageing process. However, this seems highly improbable taking into account the data available from clinical, pathological, and experimental studies, a few of which will be discussed in this chapter.

In summary, current knowledge indicates that the chaperonopathies of ageing are not only a hallmark of senescence but also a key causal factor in the physiological decline that occurs with it. The complexity of the problem concerning the role of chaperones in ageing is compounded by the fact that ageing is usually accompanied by pathological conditions (e.g., certain types of cancer, cardiovascular disorders, diabetes, and neurodegenerative diseases), that although more frequent in the elderly are not unique to them, and in which the involvement of chaperones is unknown or not fully elucidated yet. What is the role of chaperones in the pathogenesis of these diseases? Do chaperones contribute to the pathogenesis or, contrarily, do they slow down the progress of the disease and/or counteract its effects as one might expect of normal chaperones? If the latter is the case, does the appearance as age increases of defective, senescent, chaperones contribute to the onset and progression of age-related diseases?

In conclusion, no matter how one looks at the issue of chaperone involvement in the process of ageing, it is always clear that chaperones, normal or damaged in the course of ageing, are important players in senescence and in the pathological conditions associated with it. Normal chaperones are important in cell physiology at all ages, particularly in protein homeostasis, and play critical roles as anti-stress and anti-disease mechanisms. Consequently, defective chaperones are most likely implicated, at least as an auxiliary factor, in the development and progress of senescence and of its associated diseases, many of which are aggravated by stress.

The chaperonopathies of ageing are typically described as quantitative variations in their levels in cells and fluids, and as post-translation modifications (Macario and Conway de Macario, 2005). These variations and molecular modifications have been observed for various members of the chaperone families and their association with old age and age-related diseases have been abundantly reported and reviewed (Bhattacharyya et al., 2007; Kumar et al., 2007; Macario and Conway de Macario, 2001a, 2002, 2005, 2007b; Soo et al., 2008; Soti and Csermely, 2003, 2007a, b). However, data are scarce that would demonstrate a direct link between any given chaperonopathy and a defined molecular or cellular event typical of senescence, or between any given chaperonopathy and a specific disease associated with ageing. This shortage of definitive information on the exact role of chaperonopathies in the ageing process and its related pathologies is most likely due to the fact that the field is

relatively new: there has not been enough time to investigate chaperone participation in the ageing process and in disease mechanisms because the functions of chaperones are only now being more generally understood beyond experimental models, and because the chaperonopathies were identified as defined pathological entities only recently.

As mentioned above, studies pertinent to chaperones and ageing have been reported over the last several years, and information exists pertinent to members of the various families of these proteins. In this article we will concentrate only on Hsp60 and Hsp10 often grouped with the chaperonins. We will examine some of the pathologic conditions associated with ageing in which the chaperonins have been investigated and could be involved. We will omit cancer, even if certain forms of it are more frequent in the elderly, because the roles of Hsp60 and Hsp10 in malignancies have been discussed in a recent publication (Cappello et al., 2008).

MOLECULAR VERSUS CLINICAL CHAPERONOPATHIES

A defective chaperone molecule, a chaperonopathy at the molecular level, can cause a pathologic condition with clinical, anatomic, and laboratory symptoms and signs, i.e., a clinical chaperonopathy, amenable to detection and quantification. The abnormality or alteration of a chaperone molecule, i.e. a molecular chaperonopathy, can also be detected and assessed quali- and quantitatively. Alterations (e.g., mutations) in the protein-coding region of chaperone-encoding genes are typical of the genetic chaperonopathies (Macario et al., 2005; 2007c). Structural alterations in the chaperone proteins can then be of genetic origin, but they can also originate in post-transcriptional and post-translational aberrations (Bhattacharyya et al., 2007; Kumar et al., 2007; Macario and Conway de Macario, 2005, 2007b, c; Soti and Csermely, 2007b). The latter post-translational modifications are significant for the ageing process since some of these modifications have the potential to greatly impair the functional ability of proteins, including chaperone molecules (Bhattacharyya et al., 2007; Kumar et al., 2007; Hawkins and Davies, 2001; Macario and Conway de Macario, 2007c; Soti and Csermely, 2007b). Foremost among these modifications are those caused by oxidation, which can be predicted to be frequent because of the recurrence of oxidative stress as age increases (Brennan and Kantorow, 2009; Cloos and Christgau, 2004; Ksiazek et al., 2008; Soti and Csermely, 2007a, b; Squier, 2001; Stadtman, 2004; Ungvari et al., 2008).

Another factor whose importance is growing in the appreciation of clinical and basic researchers is represented by anti-chaperone auto-antibodies. These antibodies could bind the corresponding antigen in a chaperone molecule and thus impair or block altogether the functions of the chaperone, or cause immunoprecipitates with a pathological impact on cells and tissues. These types of situations dependent on the occurrence of anti-chaperonin auto-antibodies must be carefully scrutinized with regard to the ageing process and its associated diseases because the prevalence of autoimmunity and the frequency and titre of auto-antibodies tend to increase with

age (Hasler and Zouali, 2005; Liang and Gabriel, 2007; Nilsson et al., 2006; Prelog, 2006).

CHAPERONINS: EVOLUTION, GROUPS, LOCATION, GENES, STRUCTURE

Chaperonins are a subset of chaperones that have been conserved during evolution, which probably indicates that they play essential roles in the cell. Chaperonins are classified in two groups: Group I molecules are found in bacteria (e.g., GroEL) and eukaryotic organelles (e.g., mitochondrial Hsp60 also called Cpn60), while Group II representatives are present in archaea (e.g., thermosome subunits) and eukaryotic cytoplasm (e.g., CCT or TriC subunits) (Macario and Conway de Macario, 2000, 2001b; Macario et al., 2004; Large and Lund, 2009). At least one gene for Group I (Hsp60) and nine for the canonical Group II (CCT subunits) chaperonins are known to occur in the human genome (Hansen et al., 2003; Mukerjee et al., 2008).

Hsp60 and its co-chaperonin Hsp10 (or Cpn10), henceforth referred to as chaperonins for the purposes of this article, are the eukaryotic homologues of the bacterial GroEL and GroES, respectively, to which they are evolutionarily related. Most of what we know about the structure and functions of Hsp60 and Hsp10 in eukaryotes derives from studies of their prokaryotic counterparts, GroEL and GroES. In mammalian cells, Hsp60 and Hsp10 are among the most important mitochondrial molecules and play key roles in both unstressed and stressed cells (Jindal et al., 1989; Sadacharan et al., 2001). The human genes *HSPD1* and *HSPE1*, encoding Hsp60 and Hsp10, respectively, are localised head-to-head on chromosome 2 separated by a bidirectional promoter (Hansen et al., 2003). *HSPD1* mutations have been identified of which two are associated with spastic paraplegia 13 (SPG13), an autosomal-dominant, spinal-cord neurodegenerative chaperonopathy of late clinical onset, characterised by progressive weakness and spasticity of lower limbs (Hansen et al., 2002, 2007). One of these two mutations was assessed in vivo and in vitro at the gene and protein levels and found to be functionally defective (Bross et al., 2008). A third mutation segregates with MitCHAP-60 disease, which is an autosomal-recessive chaperonopathy of early clinical onset, with serious brain pathology leading to death (Magen et al., 2008).

The typical chaperoning machine in bacteria is formed by 14 GroEL molecules arranged in two stacked heptameric rings delimiting a cavity with the whole structure resembling a barrel (Braig et al., 1994; Ranson et al., 1998, 2006; Large and Lund, 2008). GroES also forms a similar ring, which does not associate with another GroES ring but with the GroEL barrel, at one of its ends, serving as sort of lid to the GroEL-complex cavity (Hunt et al., 1996). In mammals, the mitochondrial Hsp60 can form typical two-ringed barrels and also can function as a single heptameric ring, which is sufficient for productive chaperonin-mediated folding in vivo, possibly because the mitochondrial Hsp60 has a different mechanism for binding, folding, and release of substrate than the bacterial equivalent chaperoning machine

(Levy-Rimler et al., 2002; Nielsen and Cowan, 1998; Nielsen et al., 1999). Heptameric Hsp10 associates with both single- and double-ringed multimeric Hsp60 in the presence of ATP, giving rise to an "American football-shaped" molecular complex, a structure that is efficient to correctly fold other mitochondrial proteins, i.e., the client polypeptides (Azem et al., 1995). The majority of these client proteins are affected by the inactivation of Hsp60, but only a small subset of them are affected by the lack of Hsp10, suggesting that Hsp60 and Hsp10 do not always act together as a single functional unit in vivo (Dubaquie et al., 1998). Unfortunately, the full range of Hsp60-dependent protein substrates has not yet been fully delineated in humans; elucidation of this point will shed a new light on the study and identification of all the chaperonopathies due to chaperonin failure and will, in addition, significantly help to understand the molecular mechanisms participating in age-related chaperonopathies and proteinopathies.

Apart from mitochondria, Hsp60 and Hsp10 can be found in other subcellular compartments and on the cell surface (Cappello et al., 2008; Cechetto et al., 2000; Soltys and Gupta, 1996). The functions of these chaperonins in those sites that differ from the canonical intra-mitochondrial location are not yet understood, although some of them are currently under investigation.

EXTRACELLULAR CHAPERONES AND THE IMMUNE SYSTEM

Chaperones although traditionally considered intracellular proteins can also be found in the extracellular milieu as well as in the bloodstream (Cappello et al., 2008).The secretion mechanisms responsible for the chaperone translocation from inside to the outside of the cell are incompletely understood, although their levels in plasma seem partly genetically controlled (Shamaei-Tousi et al., 2007). Presence of chaperones in the extracellular space and in circulation favors contact with the immune system and thus promotes its activation, as mentioned in "Molecular Versus Clinical Chaperonopathies".

One study on psoriasis, a disease not typically starting in old age but with impact on the process of senescence, can help to illustrate the relationship of Hsp60 with the immune system. This predominantly skin disease appears to have manifestations of autoimmunity, which could play a pathogenetic role. There is information supporting the notion that the immune system can be activated via Hsp60 when this chaperonin is recognized as a ligand by Toll-like receptors (TLR) 2 and 4, and by gammadelta T-cell receptors (TCR-gammadelta), and this possibility was investigated in patients with psoriasis (Seung et al., 2007). The levels of Hsp60, TLR2, and TLR4 were assessed by immunohistochemistry in skin biopsies of 12 guttate and 12 plaque cases of psoriasis, and compared with those in five normal skins. In all specimens the number of TCR-gammadelta positive cells was determined. The levels of Hsp60 were significantly higher in guttate and plaque psoriasis than in normal skin; TLR4 was higher in guttate psoriasis as compared with plaque psoriasis and with

normal skin. TCR-gammadelta was higher in both types of psoriasis than in the normal controls, but no clear positive or negative correlation was evident between the levels of Hsp60 and those of TLRs 2 and 4, or between the levels of Hsp60 and those of TCR-gammadelta cells. Thus, it would appear that Hsp60 deserves investigation as a possible participant in the pathogenetic mechanism of both types of psoriasis with TLR4 also participating but only in guttate psoriasis and, along the same lines, it should also be determined the magnitude of the impact these mechanisms have on the process of ageing.

Another pertinent work on 60 subjects aged between 20 and 96 years showed that the serum levels of Hsp60, but not those of anti-Hsp60 auto-antibodies, declined with ageing (Rea et al., 2001). It has been reported that Hsp60 and Hsp10, when in circulation, represent a danger signal for the immune system and appear to be a key endogenous inflammatory mediator, i.e., they interact with toll-like receptors (TLR) and cause the release of pro-inflammatory cytokines and nitric oxide by immune competent cells (Van Eden et al., 2007). In view of these findings, one can assume that the reduction in the efficiency of the immune system observed as age increases is dependent at least partially on the decline in the levels of Hsp60 and Hsp10 that, according to some reports (see above), accompanies the process of senescence. However, this decline of the chaperonin levels with progression of senescence does not always occur; on the contrary, in some systems the Hsp60 levels have been found to augment with progression of senescence, as discussed later.

CELL SENESCENCE AND DISEASE

Mitochondrial chaperonins are required for a number of vital processes, so when these molecules decline in number and/or functionality, cells suffer and may die. For example, Hsp60 and Hsp10 are involved in human carcinogenesis (Cappello et al., 2008) to the extent that it has been proposed that certain forms of cancer are essentially mitochondriopathies (Czarnecka et al., 2007). Along this line of thought, and since new results have caused a shift of interest among cancer researchers and clinical oncologists toward assessing the role of cell senescence in carcinogenesis, it is likely that Hsp60 and Hsp10 play key roles in the regulation of the senescence program with impact on carcinogenesis and, also, in the pathogenesis of age-related disorders of heart, joints, skin, and the nervous system and other anatomical structures.

Cell senescence includes morphological and biochemical changes as part of a complex biologic program whereby old individuals accumulate senescent cells in their bodies. Aging cells in culture become flat and enlarged; developing extensive vacuolization and, in vitro as well as in vivo show modified secretory pathways and reduced ability to respond to stressors and to divide with growth arrest in late stages (Di Felice et al., 2005a). For instance, cultured human fibroblasts undergo molecular changes as they become older, as illustrated by the levels of Hsp60, which increase with cell age (Figure 1).

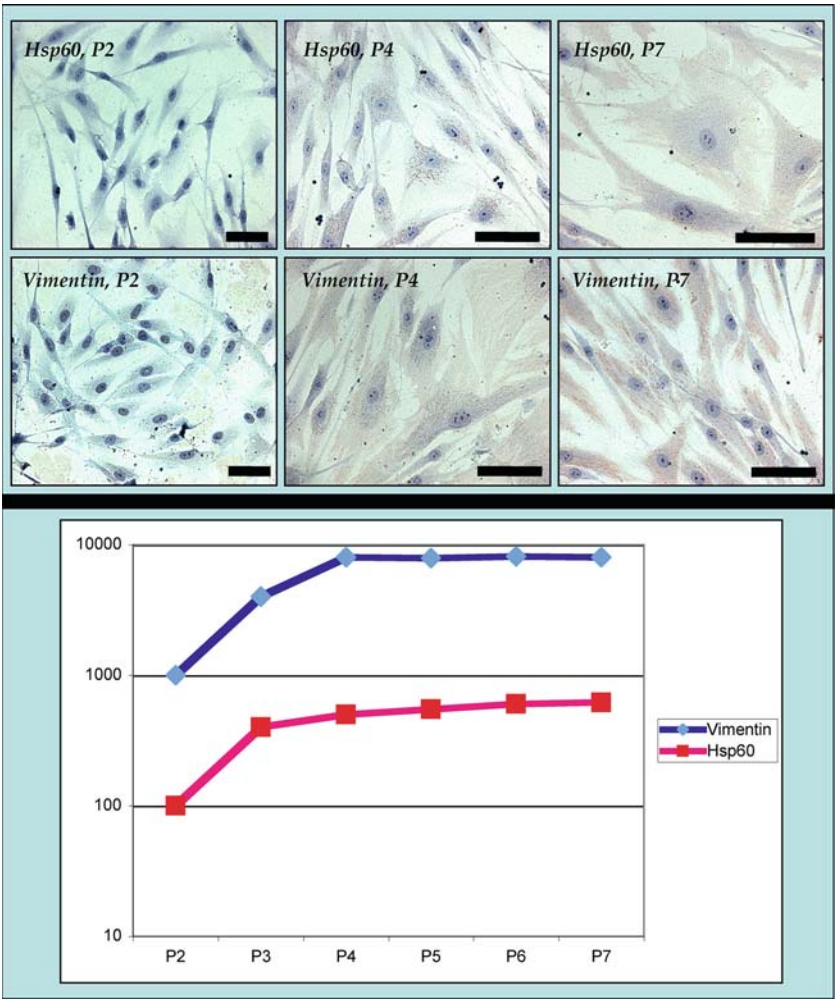

Figure 1. Hsp60 increases with the age of human fibroblasts in primary cultures. *Upper panel*: immunocytochemistry (DAB system) shows a progressive increase in Hsp60 levels during replicative senescence (RS) of primary human skin fibroblasts (PHSF) in culture, from passage (P) 2 to 7. Vimentin, a marker of RS, increases in parallel to Hsp60. Scale bar, 30 μm. *Lower panel*: computer-assisted quantification of Western blotting bands from lysates of the same cells shown in the *upper panel*. A progressive increase in the levels of Hsp60 and vimentin is apparent. Vertical axis, units of band density produced by the densitomer; horizontal axis, culture passage (P) number.

The discovery that tumor cells undergo senescence was quite unexpected, since it was assumed that they divide indefinitely. Activation of oncogenes such as Ras, Her-2, and PTEN can stimulate p53 and force cells toward senescence. In this scenario, the increase of chaperones observed in tumor cells could be seen as a

mechanism against oncogene-induced senescence (Sherman et al., 2007). This could be considered an example of chaperonopathy by mistake (Macario and Conway de Macario, 2007b) in which chaperones "help" tumor cells rather than the organism bearing the tumor, i.e., normal chaperones contribute to disease progression rather than the opposite as one would expect from the canonical functions of chaperones aimed at maintaining protein homeostasis and other physiological processes.

Some key components of the senescence process are regulated by molecular chaperones (Deocaris et al., 2006). Senescent cells are resistant to apoptosis in a p53 dependent manner but, in contrast, they can undergo necrosis after DNA damage (Seluanov et al., 2001; Sreedhar et al., 2004; Zhang et al., 2002). Senescence-induced resistance to apoptosis leads to an increase in the number of senescent cells inside many tissues, altering tissue integrity and, in turn, general health (Raffetto et al., 2001). For instance, replicative senescence of T lymphocytes has been implicated in rheumatoid arthritis (Weyand and Goronzy, 2004). Likewise, ageing of vascular cells has been implicated in the genesis of atherosclerotic lesions (Minamino et al., 2004).

Levels of Hsp60 increased in human skin fibroblasts during replicative senescence (Di Felice et al., 2005a; Lee et al., 2008) (Figure 1). A rapid increase of this chaperonin positively correlated with cell cycle progression. A positive correlation between Hsp60 increased levels and senescence in human skin fibroblasts was shown to involve interaction between Hsp60 and mtHsp70 (Kaul et al., 2006). Moreover, certain chaperones, among which Hsp60, can inhibit caspase-dependent apoptosis, conferring immortality to the cell (Nylandsted et al., 2000). Exogenous Hsp60 produced by a persistent infection with *Clamydia tracomatis* blocked the anti-apoptotic and the pro-senescence effects of the host's (endogenous) Hsp60, favoring the active proliferation of damaged cells (Di Felice et al., 2005b).

In the next paragraphs, we will review a representative sample of publications on research aimed at determining the role that Hsp60 and Hsp10 play in the pathogenesis of some age-related diseases.

ATHEROSCLEROSIS AND CORONARY DISEASE

Atherosclerosis (ATS) is a systemic disease that involves chiefly medium-sized arteries at different sites simultaneously with the lesions differing in severity and rate of progression in the various locations. Pathogenesis of ATS includes a chronic inflammation in the arteries' *tunica intima*. The inflammation is in large part promoted by macrophage proliferation elicited by accumulation of low density lipoproteins (LDL), leading to the formation of atherosclerotic plaques that consist of a fibrous cap covering a lipid-rich core. Many cell types are involved in plaque formation, including platelets, activated monocytes, macrophages, and endothelial and smooth-muscle cells. A currently accepted thesis is that atherosclerosis develops as a response to an injury of the vascular endothelium and that it is primarily a chronic inflammatory condition.

An original report on the presence of Hsp60 in endothelial cells, macrophages, and smooth muscle cells of atherosclerotic lesions was published fifteen years ago (Xu et al., 1993), and a report indicating that auto-antibodies against Hsp60 mediated endothelial cytotoxicity followed two years later (Schett et al., 1995).

In 1999, it was demonstrated that humoral immune reactions to bacterial Hsp60, such as those from *Clamydia pneumoniae* (CP) and *E. coli*, were involved in the process of vascular endothelial injury, a key event in the pathogenesis of ATS (Mayr et al., 1999). Along these lines and more recently, it was postulated that the cost paid by the body for protective immunity to microbial molecules, such as bacterial Hsp60 is the risk of cross-reactivity with human Hsp60 expressed in stressed arterial endothelial cells (Knoflach et al., 2003).

ATS could be serologically monitored by measuring anti-Hsp60 antibodies as soluble Hsp60 plays a role in activating vascular and immune cells during ATS development (Xu et al., 2000). In addition, levels of complement-activating anti-Hsp60 antibodies were found elevated in ATS related diseases (Prohaszka et al., 1999). In view of these and other similar findings, ATS has been proposed as an "autoimmune disease due to an immune reaction against Hsp60" (Wick, 2000).

Abundant data support a strong involvement in vivo of Hsp60 and, possibly also Hsp10, in the pathogenesis of coronary ATS. In a study of 35 patients with acute coronary syndromes (including unstable angina and acute myocardial infarction) and 20 healthy controls, levels of serum anti-Hsp60 antibodies were significantly higher in patients, compared to controls (Wysochki et al., 2002). In a similar study of 386 high-risk patients for coronary artery disease (CAD) and 386 age- and sex-matched controls, presence of serum antibodies to Hsp60 was associated with subsequent cardiovascular pathological events (Veres et al., 2002). In the Whitehall study, that enrolled 860 healthy subjects and 761 diabetic patients, circulating Hsp60, total cholesterol, and LDL were all high in the diabetic individuals as compared with the healthy controls (Shamaei-Tousi et al., 2007). In addition, mass spectrometry analysis showed that the circulating Hsp60 was identical to the mitochondrial counterpart but lacked the mitochondrial import peptide, so the authors suggested that this circulating protein originated in the mitochondria.

The ranges of plasmatic Hsp60 and Hsp10 concentrations were found related to genetic, biological, and psychosocial risk factors for CAD (Shamaei-Tousi et al., 2007), and the concentrations decreased with advancing age (Terry et al., 2004). High levels of anti-Hsp60 antibodies have been considered a marker, as well as a concomitant pathogenetic factor of CAD (Prohaszka et al., 2001; Lenzi et al., 2006), but these suggestions were not supported by data from other groups (Steptoe et al., 2007).

T cell reactivity to Hsp60 was measured in vitro in blood samples of 100 elderly people and 141 young men (Knoflach et al., 2007). The results indicated that Hsp60 did stimulate T cells from all individuals: the higher the reactivity of the T cells the greater the thickness of the common carotid artery but only in youngsters, indicating that specific Hsp60 immunity may have a prominent role in the very early stages of atherosclerosis.

The association between CP infection and CAD development is intriguing and the mechanism is unclear. One of the first studies on this topic showed that high levels of antibodies against both human and CP Hsp60 are independent risk factors for CAD. Other works supported the thesis that CP infection contributes to coronary atherosclerosis progression (Biasucci et al., 2003; Ciervo et al., 2002; Hoshida et al., 2005; Spagnoli et al., 2007). CP Hsp60 but not CP Hsp10 would induce maturation of monocyte-derived dendritic cells, secretion of regulatory cytokines, and enhancement of the antigen-presenting ability of dendritic cells via the T helper (Th)-1 pathway (Ausiello et al., 2006). Other researchers argued against this suggestion, reporting that anti-CP Hsp60 antibodies did not cross-react with human Hsp60 (Curry et al., 2000; Mahdi et al., 2002), and that antibodies to both human and CP Hsp60 were not associated with occurrence and severity of CAD (Hoymans et al., 2008).

Lastly, infections by *Helicobacter pylori* (Okada et al., 2007), and *Porphyromonas gingivalis* (Choi et al., 2002; Chung et al., 2003; Yamazaki et al., 2004) have also been positively correlated with a higher than average risk to develop coronary ATS, due to cross reactivity of anti-microbial Hsp60 antibodies with the human Hsp60.

At the present time, the field is confused by the number of contradictory results and, therefore, further studies are necessary to definitively clarify the roles of Hsp60 and Hsp10 and of the antibodies against them in sustaining the inflammation underlying the pathogenesis of ATS. Also, more research is necessary to identify the cell types that release the chaperonins into the extracellular space and circulation. Elucidation of these critical aspects of ATS pathogenesis will pave the way to the development of means to block the Hsp60 and Hsp10 pro-inflammatory activities if this strategy is justified by the new findings and, thus, the development of novel therapeutic, chaperonin-centered protocols aimed at slowing down or stopping ATS progression will be encouraged.

OTHER AGE-RELATED CARDIAC DISEASES

Morphological and biochemical evidence supports the notion that Hsp60 and Hsp10 play a crucial role in the preservation of heart integrity, and that these chaperonins increase during cardiac stress and are involved in the pathogenesis of heart failure. For instance, in myocardial stress due to various stressors, including ischemia and reperfusion, the blood levels of chaperonins increased (Schafler et al., 2003). However, myocardial Hsp60 did not augment after extracorporeal circulation (ECC) in patients undergoing cardiac surgery, which would indicate that ECC does not cause a stress that would increase the need for the chaperonins (Schafler et al., 2003). In contrast, oxygen deprivation for 6 h with nitrogen replacement in the saphenous vein explanted for cardiac bypass caused an increment in the Hsp60 levels, suggesting that this chaperonin is part of cellular anti-stress mechanisms (Hammerer-Lercher et al., 2001). A 5-fold increment of *hsp60* gene expression was found, by

RT-PCR, in dilated cardiomyopathy compared to normal controls, while immunohistochemistry showed that the chaperonin was chiefly localised to the interstitium (Latif et al., 1999).

It was found that *hsp10* gene overexpression inactivates Raf, ERK, and p90RSK, thus protecting cardiomyocytes from the ischemia/reperfusion-induced myocyte death (Lin et al., 2004). It was also found that *hsp10* expression is necessary to help cardiomyocytes resist apoptotic stimuli, since its downregulation resulted in ventricular cell apoptosis (Schlieper et al., 2007). Moreover, both *hsp60* and *hsp10* overexpression modulated post-translational modification of Bcl-xl, thus attenuating doxorubicin-induced cardiac muscle cell apoptosis (Shan et al., 2003).

Recently, it was shown that myocardiocytes under stress excrete Hsp60 by the exosomal pathway (Gupta and Knowlton, 2007). In addition, it was shown that Hsp60 serum levels doubled by the end-stage of heart failure and that acute myocyte injury resulted in the localisation of Hsp60 to the plasma membrane (Lin et al., 2007). The chaperonin localisation to the plasma membrane positively correlated with myocyte apoptosis and, after release of the chaperonin into circulation, with the activation of the innate immune system, promoting a pro-inflammatory state and an increase in the levels of the tumor necrosis factor-alpha (TNF-α).

Another recent study on 82 patients with left ventricular dysfunction but angiographically normal coronary arteries and 44 healthy controls revealed that the levels of circulating Hsp60 and anti-Hsp60 antibodies were higher in patients with more severe disease (Giannessi et al., 2007). Anti-Hsp60 antibody levels positively correlated with the levels of brain natriuretic peptide and with the magnitude of the left ventricular end-diastolic dimension. Hsp60 levels also positively correlated with the extent of cardiac dysfunction. From these data one may conclude that monitoring levels and localisation of Hsp60 has potential as a tool for evaluating and managing patients with heart failure.

The presence of both CP and human Hsp60 in fibrotic tissue of degenerative aortic-valve stenosis (DAVS), a chronic inflammatory process associated with ATS and hypertension, was demonstrated, suggesting involvement of the chaperonins in valve degeneration (Skowasch et al., 2003). In another study of DAVS, it was found coexistence of *hsp60* gene expression with T lymphocyte infiltration, adhesion-molecules gene expression and neoangiogenesis, all indicators of an active immunomediated process occurring in the final stage of the disease (Mazzone et al., 2004). A study on hypertensive patients, with or without target organ (heart, blood vessels, kidney) damage and associated clinical conditions did not show any difference in serum anti-Hsp60 antibody levels between these groups and the controls with normal blood pressure (Jastrzebski et al., 2006). In contrast, both Hsp60 and Hsp10 levels were increased in response to chronic atrial fibrillation (Schafler et al., 2002a, b, 2003). Hsp60 levels were also positively correlated with the degree of atrial myolysis, the higher the chaperonin levels the greater the myolysis, as shown by another study on 17 patients with atrial fibrillation and 7 healthy controls (Yang et al., 2007).

In conclusion, all these data strongly support the notion that Hsp60 could become an important marker for diagnosing and monitoring cardiovascular diseases in the

elderly. Unfortunately, no reports on the levels of Hsp10 in the same groups of patients in which Hsp60 was assessed are available to our knowledge. Information on Hsp10 could not only prove useful to understand its role in the pathogenesis of heart failure but, also, to develop diagnostic strategies based on this molecule as it has been suggested for cancer patients (Cappello et al., 2007, 2008).

NEURODEGENERATIVE DISEASES

Since Hsp60 plays a role in the pathogenesis of ATS and myocardial infarction, it can be inferred that by similar mechanisms this chaperonin is implicated in pathological conditions of the central nervous system (CNS), namely those pathologies that are caused by cerebrovascular disorders such as stroke. Furthermore, Hsp60 participates in other conditions of the CNS that do not include as the primary mechanism a failure of blood circulation due to ATS. Examples of the latter are the neurodegenerative disorders.

One of the early studies on Hsp60 in normal and pathological nervous tissue showed that this chaperonin is expressed chiefly in glial cells, astrocytes above all (Martin et al., 1993). Hsp60 appeared in a punctate pattern when revealed by immunohistochemistry, suggesting a mitochondrial localisation. This pattern appeared mainly in subjects without neurological disease while, in contrast, it was reduced in pathological specimens with the neurofibrillary tangles of Alzheimer's disease (AD) and the Lewy bodies of Parkinson's disease (PD). The authors concluded that: (a) the chaperonin could be a good morphological indicator of the "normality" of unaffected cells in brains of patients in areas not damaged by the disease; and (b) the pathogenetic significance of Hsp60 present in protein deposits of AD, PD, and also Huntington's and prion diseases would be low.

Interestingly, it has recently been found that human Hsp10, and bacterial and eukaryotic homologues can form typical amyloid fibrils under acidic, unfolding, conditions (Yagi et al., 2008). However, the fibril's core structure of the bacterial fibrils was different from that of the eukaryal counterparts, most likely due to small differences in amino-acid sequences over very short segments of the Hsp10 proteins tested.

Other researchers showed that Hsp60 expression in cultured human adult astrocytes (primary cultures) was induced by cytokines, such as interleukins IL-1β, IL-4, IL-6, and IL-10, and TNF-α (Bajramovic et al., 2000). The authors postulated that Hsp60 could play an important role in the pathogenesis of a number of nervous system diseases, particularly those with an autoimmune mechanism.

Hsp60 and other stress proteins were found elevated in lymphocytes from AD patients compared to controls (Calabrese et al., 2006a). The authors suggested that, by analogy, some stress-responsive genes could become useful targets for novel cytoprotective strategies in neurodegenerative disorders. Hsp60 was found to protect mitochondrial respiratory-chain enzymes of neurones from the toxic effect of

beta-amyloid (Veereshwarayya et al., 2006). Consequently, Hsp60 deficiency could have a deleterious impact on mitochondrial biogenesis, integrity, and function.

Mitochondrial dysfunction with impaired oxidative phosphorylation and ATP synthesis, increased production of reactive oxygen species, and apoptosis are all events that underlie the pathogenesis of Pelizaeus-Merzbacher disease. A form of this disease appears as a severe autosomal, recessive neurodegenerative disorder associated with greatly decreased brain myelinization and with leukodystrophy, recently classified as a mitochondrial Hsp60 chaperonopathy (Magen et al., 2008). Thus, Hsp60 can be included in the list of proteins involved in myelinogenesis with a potential role, when defective, in the generation of age-related neurodegenerative disorders.

Overexpression of *hsp60* and *hsp10* genes was observed in experimental subarachnoid haemorrhage in rats, possibly as a protective mechanism (Satoh et al., 2003). In an experimental model of endotoxemia, Hsp60 redistribution from mitochondria to cytosol in neurons of the rostral-ventral medulla of brain stem inhibited apoptosis and the brain stem death that would have followed in the absence of the chaperonin (Chang et al., 2006, 2007).

In conclusion, considerable amounts of data suggest a direct and important participation of the chaperonins in the pathogenesis of neurodegenerative disorders, chiefly as neuroprotective agents. However, more research is needed to completely elucidate to what extent Hsp60 and Hsp10 contribute to pathogenesis when defective or to protection when normal, and to clarify the molecular mechanisms by which these chaperonins affect the initiation, and/or modify the course, of neurodegenerative disorders.

DEGENERATIVE JOINT DISEASES

Hsp60 and Hsp10 have been implicated in the pathogenesis of degenerative joint disease, both in young and elderly people. For instance, Hsp60 was found by immunohistochemistry within the synovial tissue of rheumatoid arthritis (RA) patients, with higher levels and a different cellular distribution in comparison with normal synovial tissue (Krenn et al., 1996). RA is an autoimmune disease with pathogenesis and outcome influenced by the balance between the activities of Th-1 and Th-2 cells. Th-1 activation induced secretion of pro-inflammatory cytokines, such as IL-1 and TNF-α, by RA synovial-fluid mononuclear cells with consequent cartilage damage, whereas Th-2 activation promoted secretion of IL-4, inhibited Th-1 activity, and diminished inflammation and cartilage damage (van Roon et al., 1995). Interestingly, mycobacterial Hsp60 activated synovial-fluid mononuclear cells and suppressed cartilage proteoglycan synthesis, contributing to cartilage damage in RA (Wilbrink et al., 1993). This Hsp60 effect was dependent on the production of IL-1 and TNF-α. In contrast, human Hsp60 stimulated Th-2-induced production of IL-4, whereas Hsp60-reactive Th-1 cells released lower amounts of IL-1 and TNF-α compared to non-Hsp60-stimulated cells (van Roon et al., 1997).

It has been postulated that a humoral response against bacterial Hsp60 (exogenous chaperonin) could elicit a cross-reaction against the infected-host's Hsp60 (endogenous chaperonin) and other antigens in RA synovial tissue, thus perpetuating the local inflammatory and destructive processes (Krenn et al., 1996; Rudolphi et al., 1997). All together these data, confirmed also in experimental models of adjuvant arthritis (Ramage and Gaston, 1999), suggest that human but not bacterial Hsp60 could contribute to suppression of inflammation as a chaperone but, unfortunately, the chaperonin could serve as an antigen in the pathological lesions that would attract antibodies and, thus, contribute to inflammation and tissue destruction. All these considerations are to be taken into account when thinking of the potential for therapeutic uses of Hsp60.

Hsp10 also possesses anti-inflammatory and immunomodulatory properties via inhibition of TLR signal pathways (Johnson et al., 2005). Hsp10 inhibited the TLR4-mediated induction of nuclear factor kappaB (NFkB)-activation by lipopolysaccarides (LPS) and, also, inhibited the production of IL-6 and TNF-α by peripheral-blood mononuclear cells (Johnson et al., 2005). A double-blind clinical trial showed that intravenous administration of Hsp10 twice a week inhibited IL-1, IL-6, and TNF-α production between day 28 and day 56 (Vanags et al., 2006). The optimal dose range and route of administration were not determined.

In conclusion, both Hsp60 and Hsp10 seem to actively participate in synovial tissue generation and preservation, and also in the mechanism of synovial pathology, in conjunction with antibodies. However, the extent of involvement of the two chaperonins in the pathogenesis of senescence-related degenerative joint disorders and their precise mechanisms of action and of interaction with other molecules need to be further investigated. This investigation ought to provide key information for designing therapeutic means based on Hsp60 and/or Hsp10, using the chaperonins themselves either as the therapeutic agents or as targets for anti-chaperonin compounds. The latter anti-chaperonin agents would be indicated when a pathogenetic activity of the chaperonins is demonstrated whose inhibition would reduce cell and tissue injury (Cappello et al., 2007; Macario and Conway de Macario, 2007a).

OTHER PATHOLOGIES

Hsp60 and Hsp10 levels and the presence of anti-chaperonin auto-antibodies have been studied in relation to onset and progression of age-related diseases other than those discussed in the preceding sections of this Chapter. Some examples follow.

Diabetes. An association was found between increased auto-antibody levels against human Hsp60 and occurrence of type-2 diabetes in subjects carrying the -174C allele IL-6 gene polymorphism, as compared with individuals without the polymorphism (Mostafazadeh et al., 2005).

Skin. Increased Hsp60 in the forearm skin of elderly subjects was found in comparison with young individuals, while other Hsp did not show differences (Gromov et al., 2003). The authors postulated that the chaperonin-level changes with age

are the consequence of the mitochondrial oxidative stress characteristic of cell senescence.

Lungs. Variations in the levels of Hsp60 and Hsp10 were found to parallel chronic obstructive pulmonary disease (COPD) progression, but no such variations were correlated with the severity of COPD in smoking patients with bronchial non-small cell carcinomas (Cappello et al., 2006).

Glaucoma. The levels of auto-antibodies against human Hsp60 and of antibodies against bacterial Hsp60 have been investigated in glaucoma patients. In one study, it was found that occurrence of serum auto-antibodies against human Hsp60 did not correlate with intraocular pressure (Wax et al., 1998). In another study, consisting of a cross-sectional examination of 162 Japanese and American patients, it was found that serum antibodies against both human and bacterial Hsp60 were at higher levels in glaucoma patients compared to controls (Wax et al., 2001). However, no correlation was apparent between levels of antibodies in serum and severity of disease, suggesting that the antibodies do not play a direct pathogenetic role in glaucoma and, consequently, serum antibody measurements in this disease should be considered of low priority for diagnosis and assessing prognosis.

CONCLUSIONS

A number of studies have shown a positive correlation between longevity and capacity for mounting a strong heat shock response, implying that chaperones are key components of adaptive mechanisms for survival (Garigan et al., 2002; Hsu et al., 2003). Unfortunately, chaperone levels and/or functionality generally decrease with age. Chaperones, e.g., Hsp60 and Hsp10, could be overloaded by the increasing demand due to the accumulation of damaged proteins that occurs during senescence, which would result in a widespread chaperoning deficiency and lead to the onset of degenerative and other age-related diseases (Csermely, 2001; Soti and Csermely, 2003). A shift in the balance between misfolded proteins and available, free, chaperones in ageing organisms can bring about defects in signal trasduction, protein transport, cellular organization, and immune functions (Soti and Csermely, 2003, 2007b).

All the information available at the present time indicates that Hsp60 and Hsp10 are key factors for protein homeostasis and cell survival. Therefore, it should not be surprising if these chaperonins are found involved in various ways in the onset and progression of age-related diseases, such as the ones we have surveyed in this chapter and others that have been reported but we have not surveyed, or that have still to be identified and reported. In any event, for those diseases already described, it is necessary to investigate further the precise mechanisms by which Hsp60 and Hsp10 play their roles and, thus, obtain the basic information necessary for the development of therapeutic strategies aimed at diminishing the devastating effects of ageing and at controlling its associated diseases. In this regard, it has to be borne in mind that contradictory effects have been reported for these chaperonins in what concerns, for

example, inflammation: they can act as pro- or anti-inflammatory molecules depending on a number of factors. These factors should be determined for each pathological situation before deciding the type of therapeutic strategy to be used based on Hsp60 and/or Hsp10.

ROADMAP FOR THE FUTURE

The field of chaperones and ageing is maturing into a complex science, involving several specialities. Consequently, there is a risk of generating confusion with the steady accumulation of data without clear conceptual guidelines. Several issues have emerged in the last few years that define distinct tracts of research and medical endeavours. For example, the terms ageing (or aging), senescence, and longevity ought to be used consistently. There are many definitions for these words but one may simply adopt the following: ageing implies time, indicating the passage of time, usually years, from conception through death; senescence implies the changes (usually deleterious) in molecules, cells, tissues and greater-order structures due to the passage of time after the organism has fully developed; and longevity means long life by comparison with the average in any given population.

A distinction between ageing and senescence ought to be always possible but, in fact, a distinction is not always made and the terms are often used as synonyms. We have done so in this chapter, but when it was necessary to emphasize the molecular or cellular processes that accompany ageing and start after the organism has reached maturity (full development) we used senescence rather than ageing.

Another issue that needs clarification pertains to the role of chaperones in ageing and disease. On the one hand, there is the role of normal chaperones in the diseases that are associated with ageing, i.e., diseases that increase in frequency as the years go by. Presumably, normal chaperones will have a protective effect and will impede or counteract the deleterious effects of the disease. On the other hand, there is the role of defective, i.e., genetically defective or damaged due to the passage of time, chaperones in the mechanism of senescence and in the onset and progression of age-related diseases. A clear vision of these alternatives would be necessary for planning future research and for interpreting results, especially when it comes to a point in which cause-effects relationships must be distinguished from simple associations. Also, an accurate assessment of the status of chaperones during ageing has potential for early diagnosis of senescence and, thus, for the implementation of preventive and curative means, among which the administration of normal chaperones (genes or proteins), i.e., chaperonotherapy, appears as an option worth investigating.

The role of the immune system in the mechanism of senescence seems to be critical. Since the involvement of chaperones with the immune system is becoming more and more evident with new research, chaperonology and immunology will become progressively more and more interrelated. For example, the role of chaperones in immunity, auto-immunity in particular, will have to be investigated to

clarify those aspects of the senescence process that seem to be caused or enhanced by auto-immune mechanisms.

Age-dependent damage to proteins is another topic of great relevance for senescence and for the development of means to improve the quality of life during ageing. Age-relate post-translation modifications of proteins can seriously curtail or change their functions and thus give rise to the proteinopathies of ageing, a hallmark of senescence at the molecular level. A normal set of chaperones could potentially counteract the deleterious effects of proteinopathies but chaperones also become modified with the passage of time. These acquired chaperonopathies are likely to contribute significantly to senescence and, thus, lower the quality of life in the elderly and stand in the way of longevity by shortening the life span. Here, again, the potential of chaperonotherapy (e.g., chaperone replacement or supplementation) appears promising.

Changes in mitochondrial components are known to occur during ageing that lead to deleterious molecular and cellular alterations characteristic of senescence and linked to oxidative stress (Brennan and Kantorow, 2009; Calabrese et al., 2004, 2006b; Lee and Wei, 2007; Wax et al., 1998; Zuin et al., 2008). Mitochondrial chaperones are known to play important roles in mitochondrial biogenesis, preservation, and function. It follows that the study Hsp60 and Hsp10 and other mitochondrial chaperones, is likely to become a very rewarding activity for understanding how these molecules change with ageing, and for determining the role that defective chaperonins and chaperones (chaperonopathies of the mitochondrion) play in senescence and in age-associated diseases.

Lastly, chaperones, particularly Hsp90, have been found to be involved in telomerase assembly and activity (Forsythe et al., 2001; Keppler et al., 2006). It is likely, then, that chaperones participate in determining duration of life. It remains to be established if the chaperonins Hsp60 and Hsp10, particularly those outside the mitochondria, also participate in telomerase complex formation and/or other telomerase-related functions. Given that certain chaperones do participate in establishing telomerase activity, the important issue to investigate is what happens when one or more of the pertinent chaperones are deficient. What would be the impact on life duration of a chaperonopathy affecting a chaperone dedicated to establishing telomerase complex activity?

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