Role of heat shock proteins 70/90 in exercise physiology, exercise immunology and their diagnostic potential in sports

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Running head: Heat shock proteins 70/90 in Sports Medicine

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Abstract

Heat shock proteins (HSPs) are molecular chaperones facilitating the unfolding or folding of secondary structures of proteins, their client proteins, in cellular stress situations. Various internal and external physiological and mechanical stress factors induce a homeostatic imbalance, followed by an increased expression of HSP70 and HSP90. Exercise is a stress factor, too, and its cumulative physiological perturbation manifests at a cellular level by threatening the protein homeostasis of various cell types. Consequently, an increase of HSP70/90 was described in plasma and mononuclear cells, various organs and tissues, such as muscle, liver, cardiac tissue, and brain, after an acute bout of exercise. The specific response of HSP70/90 seems to be strongly related to the modality of exercise with several dependent factors such as duration, intensity, exercise type, subjects' training status and environmental factors, e.g. temperature. It is suggested that HSP70/90 play a major role in immune regulation and cell protection during exercise and the efficiency of regeneration and reparation processes. During long-term training, HSP70/90 are involved in pre-conditioning and adaptation processes that might also be important for disease prevention and therapy. With regards to their highly sensitive and individual response to specific exercise and training modalities, this review discusses if and how HSP70 and HSP90 can be applied as biomarkers for monitoring exercise and training.

Keywords: stress proteins, inflammation, biomarkers, recovery

Key points:

- Heat shock proteins (HSPs) are molecular chaperones facilitating the unfolding or folding of proteins in cellular stress situations such as exercise.

- HSP70/90 concentration in plasma reflects a sensitive, holistic and individual response to various physiological changes during exercise and recovery.

- Specifically athletes' capacity for a controlled downregulation of HSP concentration after exercise might be an important physiologic signal for a successful individualized regeneration process.

Table of Content

1. Introduction

Exercise is a physiological stress factor that has been shown to affect the levels of heat shock proteins (HSPs). In general, HSPs are up-regulated in response to cellular stress as needed in order to stabilize cellular functions and ensure survival. Moreover, HSPs, specifically HSP70/90, have an immunological impact, which seems to be some more differentiated and reflects a bi-directionality of HSP response (13). On the one hand, HSP70/90 were up-and properly down-regulated in order to control acute inflammatory conditions in parallel to its' pro-and anti-inflammatory chronology. On the other hand, during conditions of chronic inflammation or excessive stress, accumulation of HSP70 and 90 seems to aggravate inflammation. Therefore, HSPs have been classified as potential therapeutic targets during inflammatory diseases (12).

During acute exercise, various HSPs, such as HSP70 and 90, are up-regulated in various organs and tissues, such as blood, followed by a downregulation in the period of recovery. The term ‚recovery' means the physiological status after exercise as the body returns to homeostasis, its normal stable and physiological resting state. Both proteins are suggested to play a role as mediators of fatigue and biochemical stress sensors. Implicating their role as universal stress sensors, exercise represents only one of various cellular stressors offering a holistic view on the factor ‚stress'. Accordingly, the combination of different stressors, such as exercise and lifetime stress, or exercising during hot climate conditions, seem to aggravate HSP accumulation (19). Consequently, there is a higher physiological effort for the resolution of the stressful situation. In addition, in case of uncontrolled or chronic stress, such as conditions of chronic inflammation, the body's ability for back-regulation to homeostasis is threatened or exceeded which might implicate pathological conditions, such as overtraining (65).

Based on this assumptions, the current review aims to discuss these differential roles of HSP70 and 90 in exercise physiology with a specific focus on the bi-directional impact of these HSPs during inflammation and exercise stress. Here, it seems to be of specific importance that HSPs are not only up-regulated during stress, but also properly down-regulated during resolution of the stressful situation. Regulation of HSP70/90 in blood offers the opportunity to use these molecules as stress

sensors and biomarkers. In this regard, HSPs might not only offer possibilities for quantification of stress, but also for monitoring recovery processes holistically (4). Using such sensor proteins as biomarkers during exercise and recovery might help to control training processes adequately in accordance with individual abilities. In parallel, other interfering stressors, such as situations of psychological stress, are also considered, having in mind the picture of a “stressed athlete”. When training stimuli are wrongly observed due to a lack of systematics, and athletes are additionally burdened by lifetime stressors, there might be an inadequate balance between stress and recovery processes (32, 44, 76, 109). Hence, situations of chronic stress and overtraining might occur, which may be of special interest for monitoring (65). Biomarkers would be of great value for checking and controlling the internal load condition, specifically, with regard to their objectivity, sensitivity, reproducibility and automation of the monitoring processes (7). It, therefore, seems desirable to exploit further potential biomarkers for sports practice beyond the known markers. Muscle enzymes and hormones are identified as stress-sensitive biomarkers in the blood plasma, inter alia creatine kinase (CK) or lactate dehydrogenase (LDH), are used in specific settings for training control and monitoring of regeneration. However, these parameters have been shown to have a high variance in response to a standardized training program. The partly cost-intensive measurement effort and lack of reliability of these enzymes adversely affects the application in sports practice (27, 32, 66, 109). Thus, there is a requirement of innovative biomarkers that reflect an athlete stress level.

Especially HSP70 and 90 are key factors after stress encounter. They are both expressed in response to inherent physiological changes, for example after exercise (84). The level of plasma HSP70/90 during exercise increases individually dependent on intensity and duration. Exceptionally the enormous release of HSP70 and 90 during high-load exercise is speculated to contribute to fatigue sensations (34). Thus, both HSPs are considered as potential biomarkers, which can give a variety of information on individuals’ stress processing and the course of recovery processes. In the process, other interfering stress factors are also considered, culminating a holistic reflection of stress experiences in athletes.

2. The Chaperones: A servant network for client protection.

HSPs are molecular chaperones in cells that help their client proteins in the unfolding or folding of their secondary structures in cellular stress situations (106). HSPs are divided into six families (HSP100, HSP90, HSP70, HSP60, HSP20 and small HSPs). In figure 1 prominent are HSP90/70 while HSP100 and the other HSPs separated into group I and group II HSPs. Physicochemical stress or single-point mutations in the genome, which lead to amino acid exchange, cause non-functional or denatured proteins; HSP70/90 can determine the fate of the proteome pool under stress conditions (43). HSPs are essential chaperones that actively organize the correct folding of newly synthesized or denatured proteins in cells, thereby preventing the formation of aggregates (16, 50, 62). They do not have any information about the correct folding of the clients, but through their binding, they support the proteins in their folding process by avoiding aggregation or faulty interactions with other molecules. In a broad variety of species, HSPs are involved in signal transduction, in the control of the cell cycle, in stress management and in the transport of proteins (14). Not only is the expression of some of these genes highly heat-inducible, but it is also induced by other environmental factors such as UV radiation, oxygen deficiency, or the presence of ethanol or heavy metals (51, 67, 87). A major aim in HSP research is to understand the different roles of HSPs and their controlled response to stress (45, 105). Stressful situations, which can occur from cancer, extreme sport, lifestyle, or ageing, can produce a huge amount of damaged proteins (72) (Figure 1). The concentration is at approximately 200-300 g/L, in comparison to a protein blood concentration of 80 g/L, demonstrating that there is a requirement to protect and stabilize the proteome. For all regular processes and for cells, it is energetically more advantageous to recycle proteins than to synthesize them anew (20). Several inhibitors have been developed against HSP90 and HSP70 to hinder disease related stress response. HSP90/70 have ATP-binding and client binding sites. HSP90 has high affinity binding sites for co-chaperones, e.g. activating AHA1, inactivating p23, p50, CDC37 and HSP70 and client proteins whereas HSP70 function is dependent from HSP40 and NEF (Figure 1).

149 The human superfamily of HSP70 consists of 13 members, four of which account for the largest share
150 (15, 18, 30, 97). These include: the Grp70 located in the endoplasmic reticulum, the mitochondrial
151 mtHSP70, the constitutively expressed HSC70, and the stress-induced HSP70. HSC70 is involved in the
152 folding of newly synthesized proteins, the transport of proteins across the cell membrane, and the
153 assembly of multiprotein complexes. As a result of proteotoxic stress, the protein is present at higher
154 levels and is available to the cell as an active folding aid (18). In the promoter region of heat shock
155 transcription factor-1 (HSF1), the regulation of the expression of heat shock elements (HSEs) is
156 controlled by means of external stress factors.

157 HSP70 captures client proteins and binds directly to exposed hydrophobic regions of unfolded and
158 misfolded proteins, preventing their aggregation and giving them the chance to refold in the absence
159 of other proteins (15, 30, 86, 97). HSP70 seems to be the most conserved protein in evolution so far
160 (97). There are highly conserved sequences in the relevant functional sections, such as the ATP
161 binding sites in the N-terminal domain, for one. The HSP70 superfamily has additional roles in the
162 field of cell preservation, such as the dissolution of protein complexes, the transmission of cell-cell
163 signals and the transport of proteins between subcellular components and organelles (30).

164 **3. Physiological relevance of HSP 70/90**

165 Evolutionarily, HSP70/90 families are highly conserved and can be found in all cells from archaea,
166 eubacteria, and eukaryotes (26, 40, 58, 102). In general, HSP90/70 are essential for eukaryotic cells,
167 whereas in eubacteria recent results show a dependency on environmental adaptation (58). Studies
168 in *Saccharomyces cerevisiae* have found that 10% of all cellular proteins are directly or indirectly
169 dependent on the proteins of the HSP90 family. They are, for one, proteins that need the chaperones
170 to fold, stabilize or activate, but also co-chaperones that regulate the functions of HSP90. The
171 activation of the chaperoning system begins with the activation (trimerization and phosphorylation)
172 of transcription factor HSF1 and influences basic cellular maintenance processes and molecular
173 mechanisms like autophagy, multidrug resistance, programmed cell death, cell cycle arrest,

chromatin structure, and immune response. These functions of HSF1 intervene deeply in development and physiology (31, 46, 73).

The HSP90 family can be divided into five subfamilies; four of which are present in eukaryotes, one in prokaryotes. In eukaryotes, the HSP90 genes are part of the nuclear genome, but the four different isoforms of the eukaryotic HSP90 are present in different cell compartments. In the cytosol, there are two forms, the HSP90α (inducible form) and the HSP90β (constitutive form). They are to 85% identical and were created by gene duplication around 500 million years ago (102). The two proteins are summarized as the HSP90A subfamily and are the best-studied proteins of the HSP90 family. Under stress-free conditions, HSP90 accounts for 1-2% of cytosol proteins, whereas stress can double or even triple their concentration in the cell (14). Another protein family is the glucose-regulated protein (Grp94/gp96 former Erp99), also known as HSP90B, located in the endoplasmic reticulum (ER) (also referred to as gp96), which belongs also to the HSP90 family. Except for fungi, the HSP90 family protein Grp94 is present in all eukaryotes. Grp94 supports the cell during stress and helps in breaking down misfolded proteins on the ER-associated pathway. Known Grp94-dependent proteins include insulin-like growth factor 2 (IGF-2), immunoglobulins and other signal transduction-mediating pattern recognition receptors (PRRs). The third subfamily contains the protein tumor necrosis factor receptor associate protein 1 (TRAP-1) (105), which protects the mitochondrion from oxidative stress. This HSP is most similar to the bacterial member of the HSP90 family, the high temperature protein G (HtpG) (119). However, they are unlikely to be endosymbiotic. It seems as if TRAP-1 separated early from the other three eukaryotic families and thus is very similar to the HtpG. TRAP-1 is the only representative with a unique LxCxE motif, which provides protein-protein interactions and is not found in the other members of the HSP90 family. During evolution, the proteins of the HSP90 family in eukaryotes have had a significant influence on the formation of phenotypes by genetic variations. The chaperones still have to fold proteins correctly, especially when they occur from genetic alterations of the DNA caused by stressors. In addition, HSP90 support many specific signaling vectors and thus lie at the interface of many developmental pathways. In case of great

environmental stress, such as pathogen infection or mechanical injury, even HSPs become inactive and phenotypical changes occur in the organism (43). Previously hidden gene variants then come to the fore. These "disadvantaged" variants are normally not present due to HSP90 buffering. In *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Danio rerio*, new phenotypes were observed after the elimination of HSP90 proteins. In the otherwise complex developmental processes, evolutionary change can thus be promoted if a new phenotype is beneficial.

4. Immunological effects of HSP 70/90

Besides their role in maintaining the integrity of cellular proteins, HSPs have an important immunologic impact. In particular, members of the HSP70/90 family have crucial functions for inducing or balancing immune reactions and regulate acute or chronic inflammatory processes by their constitutive expression or after their intracellular or extracellular accumulation (39).

The important role of HSPs as immunological signalling molecules became obvious after experimental administration of exogenous HSP, which induced a variety of immune functions that are currently exploited in immunotherapy of cancer, infectious diseases, and autoimmune diseases. These immune responses indicate that HSPs represent a kind of self-antigen which is present in and secreted by our own cells. Thus, they are recognized by our immune system and generate pro-inflammatory signals. Conversely, HSPs have been shown to induce immune-regulating effects by modulating specific anti-inflammatory pathways and anti-apoptotic effects (41). However, the immunologic impact of HSPs seem to depend on various factors such as the type of inflammatory response, the location of HSPs, their clearance as well as the type of accumulated HSPs (47).

Recent studies demonstrated a strong interaction between Toll-like receptors (TLRs) and HSPs. TLRs are the critical sensors for recognition of microorganisms which expression patterns are closely related to the immunological function of the cells (85). On the one hand, Hsp70 is known to be a ligand for TLRs. On the other hand, all TLRs, except TLR3, are known to be client proteins of gp96. Their correct folding is dependent on dimerization of gp96 in the ER lumen. Consequently, gp96

depletion results in a loss of TLRs and unresponsiveness to TLR ligands. Knock-out mice, which are deficient for gp96 expression, have an increased susceptible to bacterial infection (52, 101).

4.1 HSPs and the cross-presentation of antigens

An important immunological process, which is supported by HSPs, is the cross-presentation of antigens. Cross-presentation describes the ability of exogenous antigens to enter endogenous pathways of MHC class I molecules, followed by a priming of $CD8^+$ T cells (38). The process of cross-presentation can occur via both, an endocytic pathway and a cytosolic pathway. For both pathways, constitutive expression of HSP70 and 90 are required to translocate antigens into the cytosol (38). The process of peptide cross-presentation bound to HSPs represent a receptor-mediated pathway. HSP70 and gp96 have been shown to bind to several receptors, such as CD91 and LOX1, while HSP90/gp96 activate mainly scavenger receptor-A on antigen presenting cells (APCs). Pharmacological inhibition or deletion of HSP90 abrogates antigen cross-presentation. Accordingly, HSP70 and 90 are specific and important regulators of antigen cross-presentation (9).

4.2 Opposing effects of HSPs during inflammation

In early studies, HSPs were suggested to be exclusively intracellular proteins, implying that an extracellular increase is exclusively a result of cellular damage, injury or necrosis. These assumptions classified HSPs as “danger associated molecular patterns” (DAMPs), which represent ligands for pattern recognition receptors (PRRs) (3). PRRs are host sensors of the innate immune system, able to detect both molecules typical for pathogens, such as pathogen-associated molecular patterns (PAMPs), and DAMPs, which are associated with the components of host's cells. These molecules are generally released during cell damage or death. The classification of HSPs as DAMPS implies that their signalling is distinct from the presence of pathogens, certainly also activating pathways of the innate immune response (8).

Later studies proved that HSPs are not only passively released but also actively secreted into the extracellular environment by various cell types, such as necrotic cells, lymphocytes, natural killer cells

(NK), and tumour cells, via exosomes (25, 59, 65, 71). In particular, for proteins of the HSP70 and HSP90 (gp96) family, active signalling to stimulate the innate immune response has been proven (99).

A variety of immune cells, e.g. dendritic cells (DCs), NK cells, and macrophages, can detect HSPs, like HSP70, via CD36, CD40, and CD91 surface receptors. Interestingly, HSP70 is also recognised by T cells through T cell receptors (TCR) during a presentation with major histocompatibility complex (MHC) molecules (99, 111). For HSP90/gp96 chaperones, it has been shown that they interact with TLR2 and TLR4 molecules that can induce the activation of the NF-κB pathway, followed by the production of a variety of cytokines, such as IL-12 and TNF-α. This activation process is induced by the C-terminal peptide-binding region of HSP70 (49, 117). In most cases, HSP binding to one of these receptors induces the initiation of an activating, pro-inflammatory signalling cascade, followed by the initiation of nonspecific cytokine and chemokine production and, in parallel, upregulation of co-stimulatory molecules (Figure 2) (2). Similar processes have also been shown after treatment of THP-1 cells with mycobacterial HSP70 (49, 117). However, there is also some evidence for differential regulation processes after treatment with mycobacterial HSP70 or human HSP70. These differences might be explained by different binding regions at CD40 on macrophages and dendritic cells (DCs) (6). While mycobacterial HSP70 seems to exclusively induce pro-inflammatory signals, mammalian HSP70 not only activates but also regulates the immune response by inducing anti-inflammatory signals. However, some researchers argued that there was a complication in initial studies regarding the co-purification of lipopolysaccharide (LPS) as a contaminant in preparations of recombinant HSPs (110). This discussion remains controversial because *in vitro* studies with highly purified HSP molecules might not represent a suitable *in vitro* model for the complexity of HSP activities in a living organism. However, current studies suggest that HSPs might have both immune-stimulating and anti-inflammatory effects. These opposing roles are rationalized by the complexity of inflammatory processes, which have to be tightly regulated. Meanwhile, the production of alarm signals sharply diminishing after the pathogens or cellular debris have been eliminated and tissue homeostasis is restored (96). This may depend at least partly on the cellular location of HSPs. While extracellular

276 HSPs might serve primarily as danger signals, stimulating the immune response, intracellular HSPs
277 serve as immune regulating factors (92).

278 These findings are supported by studies demonstrating that intracellular HSP70 accumulation
279 induces the inhibition of NF-kB activation, something that has a profound implication for immune
280 activation and inflammation. It was shown that HSP70 inhibits the phosphorylation of inhibitor of kB
281 (IkBs). Other studies demonstrated that tumor-derived exosomes (TDE) -associated HSP70 activates
282 the mitogen-activated protein (MAP) kinase cascade, one of the most ancient and evolutionarily
283 conserved signalling pathways (17). In detail, it was found that HSPs mediate a suppressive activity of
284 the myeloid-derived suppressor cells (MDSCs) by activation of STAT3 and ERK, followed by IL-10
285 production (13, 90). IL-10 secretion is one of the central mechanisms proposed for the immune-
286 regulatory function of HSPs (39).

287 There is also some evidence indicating the immunological effects of HSP70 or HSP90 also depend on
288 the inflammatory milieu because HSPs are suggested as targets in chronic inflammatory diseases,
289 such as rheumatoid arthritis (115) and atherosclerosis (118). Elevated levels of HSP70 and gp96 have
290 been found in synovial fluids from inflamed joints in rheumatoid arthritis patients and in
291 arteriosclerotic lesions (61, 94). Accordingly, during situations of chronic or uncontrolled
292 inflammation, HSP-accumulation might intensify pathologic processes by stabilizing proteins, which
293 support or induce pathological processes. These situations are characterized by chronic HSP-
294 signalling, something that might evoke completely different effects than during acute or short term
295 HSP increase. In contrast, during conditions of acute cellular stress, e.g. heat shock or exercise, HSP
296 induction primarily controls immune processes, followed by a downregulation of inflammation and
297 HSP concentration (Figure 2) (11).

298 **5. HSP 70/90 response to exercise**

299 Exercise is a physiological stress factor accompanied by various inherent physiological alterations,
300 known to increase HSP70 and HSP90 concentration. Oxidative stress, modifications in temperature,
301 pH and ion concentrations, decreases in calcium concentration and intramuscular glycogen,

disturbed membrane integrity as well as glucose deprivation during and after exercise provide conditions of instability and consequent homeostatic imbalance. Also tissue hypoxia, which presence a condition of lower than normal oxygen content and pressure in the cell, might be a trigger of HSP expression (10, 55).

Consequently, an increase of HSP70/90 was described in plasma and mononuclear cells and various organs and tissues, such as muscle, liver, cardiac tissue, and the brain after an acute bout of exercise (5, 70, 98, 100). Specifically, the HSP70 response seems to strongly depend on the mode of exercise. Duration and volume of exercise, exercise type and intensity, subjects' training status and environmental factors, such as heat, have been shown to affect the extent of HSP upregulation after exercise. Most studies used endurance exercise protocols and demonstrated that higher intensities seem to more strongly upregulate HSP70 production in plasma and muscle compared to activities of moderate intensity (53, 68). However, Walsh et al. (2001) proved that endurance exercise of moderate intensity also results in a significant increase in the concentration of circulating HSP70 in humans (116). In other studies, it was shown that prolonged exercise induced a more pronounced response of serum HSP70 than shorter or more intensive exercise bouts (24). A correlation was shown between pre-exercise HSP values and the increase in HSP70 post-exercise in human muscle biopsies measured over 5–8 weeks of exercise (29). After periods of intensified training, higher basal levels of HSP70 were found, which could reflect an accumulation of HSPs after several subsequent bouts of exercise in leukocytes (23). This finding is supported by the findings that HSP70 is specifically elevated after periods of intensive exercise with only short periods of recovery in muscle tissue (54). Similar observations were made in studies with rowers, where a progressive accumulation of HSP70 was found in skeletal muscle of well-trained rowers after 3 weeks of high-intensity strength training (53). During a subsequent week of recovery, HSP70 values decreased to baseline values. Chronically elevated basal levels of HSP70 were found in cardiac tissue of trained mice. Interestingly, in these mice, an acute bout of treadmill running did not induce a further increase, suggesting a kind of pre-conditioning in cardiac tissues after training (64). In contrast, lower basal HSP70 concentrations were

shown within peripheral blood mononuclear cells of trained subjects, which might also reflect an adaptation to regular exercise (23). Accumulated Hsp70 in muscle is suggested to work as a negative feedback regulator for the inducible transcription of HSP70 genes. This might be an important mechanism to secure a controlled regulation of stress proteins and to keep the capacity for an efficient recovery. Accordingly, endurance athletes might have – except in cardiac tissue - lower HSP concentrations in tissues when compared to non-trained individuals, although increases in HSP were still observed in response to acute exercise (23). It was also proved that heat stress during exercise, e.g. during running in a hot environment, amplifies HSP70 concentration in muscle, cardiac tissue, and mononuclear cells suggesting that external heat is an additional stress factor (98). Furthermore, the type of exercise affects HSP concentration. While both, endurance and resistance training seem to similarly induce HSP70 expression, eccentric exercise after downhill running has been shown to be more efficient in raising the HSP70 response than horizontal running in muscles of rats (57).

5.1 Mechanisms of HSP expression during exercise

In response to acute exercise, transcriptional expression of the HSP70 gene is regulated primarily through heat shock transcription factor 1 (HSF1). After activation, HSF1 trimerizes and translocates into the nucleus, and binds to the promotor region of the HSP70 gene. In parallel, exercise-induced activation of the downstream-adrenergic receptor-mediated signaling kinase (Proteinkinase A) occurs, which inhibits ERK1/2 activation and therefore is permissive to increase HSP70 concentrations.

Since acute and chronic exercise elicits several physiological alterations, which might mediate HSP70/90 induction, more than one factor of homeostatic imbalance is suggested to affect HSP concentration. The increase in body temperature seems a rather obvious signal to induce the expression of HSPs. During exercise, core body temperature in humans can reach about 40°C during heavy exertion (88). That heat production is certainly a contributing mechanism for HSP expression during exercise, which is supported by the pronounced expression during physical activity in a hot environment. However, in some tissues, such as skeletal muscle, an increase in HSP70 concentration

was found without any thermal stimulus. Accordingly, the mechanisms of HSP70 expression might be tissue-specific (70, 98, 100). Studies on HSP70 expression in muscles and liver gave evidence that increased oxidative stress was associated with levels of increased HSP70 concentrations (89). The increased production of reactive oxygen species (ROS) during exercise leads to non-specific reactions with cellular components bringing about damage to proteins, lipids and nucleic acids. In muscle cells, several enzymatic defense mechanisms, which involve superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in addition to non-enzymatic antioxidants, are active in order to prevent oxidative stress. In parallel, synthesis of the protective HSP70 starts to maintain myofibrillar integrity and stabilizes cellular proteins (108). In skeletal muscle, HSP70 concentration was positively associated with compromised glucose concentrations (22). The relevance of substrate availability for HSP70 levels is supported by a set of data, demonstrating a blunted HSP response after supplementation of glucose (22).

It was also demonstrated that hypoxia is a trigger of HSP expression (10, 55). In this regard, it was shown that the activity of both HSPs and HIF-1α increased after second heat or hypoxia. This up-regulation of both factors in response to different stressors is designated as a process of cross-tolerance. Thus, HSPs lead to a kind of preconditioning, which aims to confer tolerance to subsequent different cellular stressors. In line with these findings, a repeated bout has been shown to increase the level of endogenous HSP70 as well as muscle HSP70 but to a lesser extent than after the first bout of exercise. Interestingly, before the second bout of exercise, basal levels of HSP70 were decreased compared to the levels before the first exercise bout (21, 60, 107).

Another trigger of HSP70 expression in muscles seems to be elevated levels of intracellular calcium (Ca^{2+}). Cell culture experiments using muscle cell lines showed an increased HSP70 expression concomitantly with action potentials and membrane polarization after electrical stimulation. This effect was blocked in the presence of Ca^{2+} inhibitors, indicating calcium signals as a main trigger (42).

5.2 Role of HSPs in exercise physiology

HSP70/90 expression and secretion during exercise might have multiple functions. The release of HSP70 into the blood stream might be a danger signal induced after exposure to physiological stress. Possibly, it aims to support immunological processes associated with regeneration and reparation of tissue damage, restoring tissue integrity or inducing adaptation (12). Some researchers speculate that increasing levels of peripheral HSP70 molecules participate in fatigue perception. For intracellular HSPs, a protective role against injury, such as ischemia-reperfusion damage, is supposed, which comprises the immune-regulating and anti-apoptotic potentials of HSP70/90 (34, 93). Senf et al. (2013) found that extracellular HSP70 has a role in muscle repair and fiber adaptation by restoring the recruitment of muscle cells involved in the inflammatory response (95). The expression of HSP70 in certain locations might have protective effects against death of motor neurons and muscle cells (77). In studies of Paulsen et al. (2007), an association was found between muscular force generation and HSP70 levels. Probably, HSPs function as stabilizers of disrupted myofibrillar structures is to contributethe reparation and adaptation processes by refolding denatured proteins and folding of newly synthesized proteins (80). The authors also speculated that HSP70 blunts inflammatory processes in muscle, something that might support regeneration processes. It is also speculated that HSP production contributes to long-term adaptations by preventing muscle damage after repeated bouts of eccentric exercise (“repeated bout effect”). This effect describes the phenomenon that plasma CK was elevated after exercise at the beginning of conditioning programs and decreased in response to a repeated bout of exercise (10).

With regard to the preventive and therapeutic effects of exercise, animal experiments suggest an important role of HSPs for exercise preconditioning and pre-habilitation. For example, preconditioning processes induce an increased HSP70 concentration in cardiac tissue, which reduced myocardial infarction, ischemia reperfusion injury and increased survival time during heatstroke in rats (36, 37, 56, 82). Accordingly, HSP expression is suggested to be a mechanism that may at least partially account for the preventive effect of exercise (7).

6. Biomarkers in exercise physiology

The early detection of biomarkers is very important in the case of cancer, cardiovascular disorders, and other pathological conditions, but also for monitoring normal physiological processes with the aim to analyze stress levels and understand stress limits. Biomarkers in sport physiology help to monitor training success and analyze nutrition and metabolic health, hydration status, muscle status, endurance performance, injury status, and inflammation (5). These intrinsic data help to structure training processes and should be underpinned by high accuracy and precision. Thus, these markers should give a comprising and powerful understanding of individual physiological balance (35, 103). However, this biomarker displays an appropriate evidence supporting their use, but also have crucial limitations (32). CK as a popular recovery marker mirrors a muscle fibre damage and show increased values in studies with intensive exercise bouts. However, the measures of this biomarker observe large intraindividual and interindividual variabilities, and a poor temporal relationship with muscle recovery exists (91, 114). Furthermore, biochemical, hormonal, and immunological biomarkers such as blood lactate, inflammatory cytokines, cortisol or C-reactive protein have an immense fluctuation width and are questionable to provide valid and reliable data on fatigue and recovery parameters (44, 48, 109). These value variabilities depend on factors like age, sex (74), daytime (33), and individual training status (78). From the results by many studies, it can be derived that no single parameter could represent an adequate sensitive and reproduced fatigue/recovery-status of athletes (35). Studies that were carried out with multiple biomarkers indicate that combinations are a better predictor to monitor fatigue/recovery processes than a single marker (104). Thus, it is necessary to identify more universal and consistent biochemical training markers of multiple aspects of athlete health and performance.

6.1 HSPs 70/90 as potential biomarkers to monitor training

HSP70 and 90 might represent some of these promising biomarkers for reflecting levels of exercise stress, state of systemic recovery and long term training load (63). Both markers manifests high values after acute exercises, correlate well with the fatigue status and might be predictors for the efficiency of regeneration (113).

A high sensitivity of serum HSP70 has been shown in particular for the process of regeneration. Accordingly, during recovery from exercise, several physiological processes occur, including repair processes of damaged tissues, down-regulation from inflammation, filling up energy stores and decreasing oxidative stress, all of which affect HSP70concentration (58, 74) Correspondingly, HSPs seem to be a suitable marker to monitor progress and time frame of regeneration. Following this idea, researchers suggested that HSP70 represents a signalling molecule for exercise-induced fatigue (10). Otherwise, chronically elevated levels of HSPs implicate a lack of recovery and, thus, are a kind of precursor for pathophysiological states, such as overtraining. In this regard, other studies support the idea that overreaching or overtraining can be mainly defined by an inability to recover (58). A chronic increase of HSPs might reflect a reduced space for a further up-regulation in response to an additional acute stress event. This assumption is supported by data from patients with chronic fatigue syndrome, in which acute HSP70 responses are suppressed (10). Consequently, it is speculated that the reduction of HSP concentration during physiological regeneration creates space for newly applied acute stress events (Figure 4). Considering the clinical relevance and the key role in physiological and pathobiochemical adaptations, it becomes obvious that HSP70/90 emerges as a potential biomarker for monitoring not only exercise, but specifically the course of recovery.

6.2 Classical diagnostic strategies and innovative detection options

The use of HSPs as a biomarker in tissue and bodily fluids, such as blood and urine, requires techniques for easy and fast detection. Newer results indicate that HSP90α is a good biomarker for liver cancer in the early stage using an ELISA technique for detection (28). This method might also be employed to evaluate limits for endurance exercises as well as for personalized records. The main problems are the detection limits, sensitivity and artificial quenching by foreign compounds, e.g. albumin etc. (81, 112). To circumvent limitations and to provide the highest reliable sensitivities, biomarkers need to be identified by different techniques. In general, the presence of HSPs can be monitored by immune blots, ELISA, mass spectrometry, SPR or protein microarrays, and also Raman-based detection systems (75). ELISA and related systems use the affinity of antibodies or aptamers as detection sensors while the signal is enhanced and detected electrically or optically. Due to the high

miniaturization grade, protein microarrays are a powerful tool in monitoring the presence of HSP biomarkers, using a known fixated probe (antibody, aptamer, ligand) against unknown multiple samples (69). These technologies are under continuous optimization and, in some cases, can detect picograms of biomarkers. Required are innovative technologies, which detect physiological and simple analytic biomarkers, such as DNA, miRNA, protein, peptides as well as small molecules rapidly during endurance; most of them have been endorsed by the US FDA and explored for the improvement and cost reduction of healthcare services. These technologies comprise around 664 non-invasive molecular biomarkers and the 592 potential minimally invasive blood molecular biomarkers available for monitoring, diagnostics or theragnostic purposes (83). Technologies like microfluidics or microelectrical sensors for protein biomarker detection can be developed for personalized applications as well for point of care (POC) diagnostic of disease markers, which, in future, could provide reliable and low-cost sensors for monitoring the physiological status (1, 79).

7. Perspectives

Current knowledge suggests that both HSP70 and HSP90 might have important functions for cytoprotection, immune regulation, regeneration, and adaptation processes during exercise and training. Considering their sensitive and individualized response to various physiological changes during exertion and regeneration, these proteins can be considered as biomarkers to identify the specific response of an individual to exercise, additional stress factors, and for monitoring stress/recovery cycles during training periods in athletes. In this framework, specifically the capacity for a controlled downregulation of HSP concentration might be an important physiologic signal, since pathophysiological, immune-dysregulated or overstrained systems seem unable to regulate HSP expression back to baseline level (65). Accordingly, HSP70/90 expression may also be considered as potential markers for the early detection of overtraining syndromes in athletes. Similarly, the induction of these proteins in different tissues and cell types after exercise seems to be a physiological mechanism, which mediates some of the preventive and therapeutic effects of regular exercise for the treatment of various diseases. Concurrently, their expression might be a suitable

marker for finding an effective dosage to calculate exercise duration, intensity and frequency in patients with differential exercise capacities

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800

801 **Figure legends:**

802 Figure 1: Composition of the human chaperome. Stress affects expression levels of HSP90/HSP70
803 with manifold consequences on the proteome therefore are prominent ATP-dependent stress
804 markers in many diseases and embedded in a network of cochaperones (Hop, p23, CDC37, AHA1,
805 HSP40). Targeting HSPs by inhibitors can stop the folding machinery directly and hinder thereby
806 survival of diseased cells. (HOP: HSP70-HSP90 Organizing Protein, AHA1: Activator of HSP90 ATPase,
807 NEF: nucleotide exchange factors).

808 Figure 2: Effects of chronic cell stress compared to conditions of acute stress on HSP70/90 regulation
809 and inflammatory signaling pathways in monocytes (NFκB: nuclear factor-κ B, IκB: inhibitor of κ B, P:
810 phosphorylation).

811 Figure 3: Overview of increased expression of HSPs in different organs and tissues during acute
812 exercise and their physiological triggers, exercise-dependent factors and potential physiological
813 relevance.

814 Figure 4: Concept of HSP regulation during pathophysiological conditions (e.g. chronic stress) and
815 during exercise training. During condition of chronic stress, HSP concentration progressively
816 increases, which supports a condition of chronic inflammation. The body loses its ability
817 downregulate HSP concentration in response to internal or external stressors. During conditions of
818 regular exercise training, HSP concentration is up-and downregulated, indicating a stress and
819 recovery process. Accordingly, a downregulation of HSPs is suggested to create “space” for an
820 effective upregulation during additional or repeated stressful stimuli.

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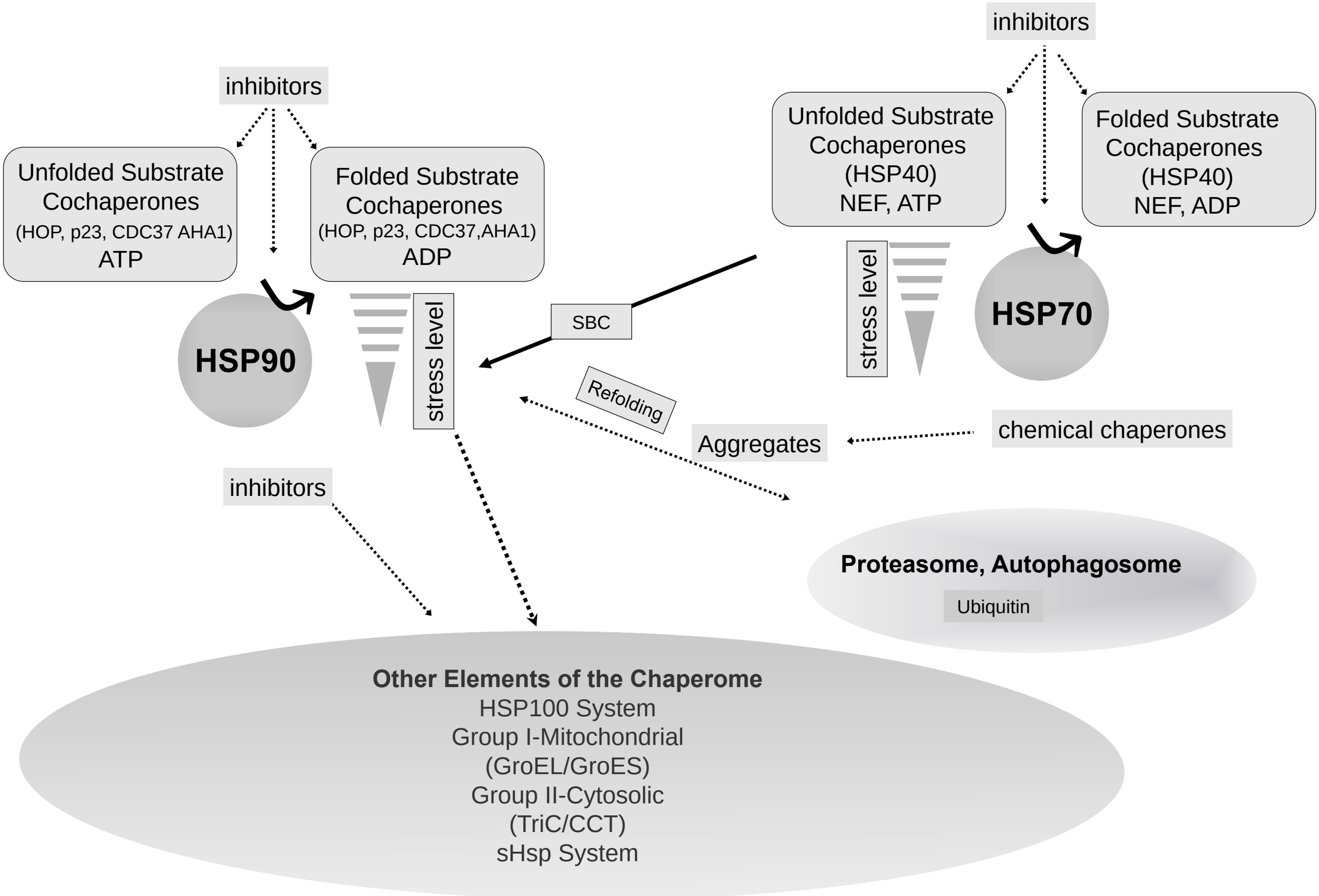
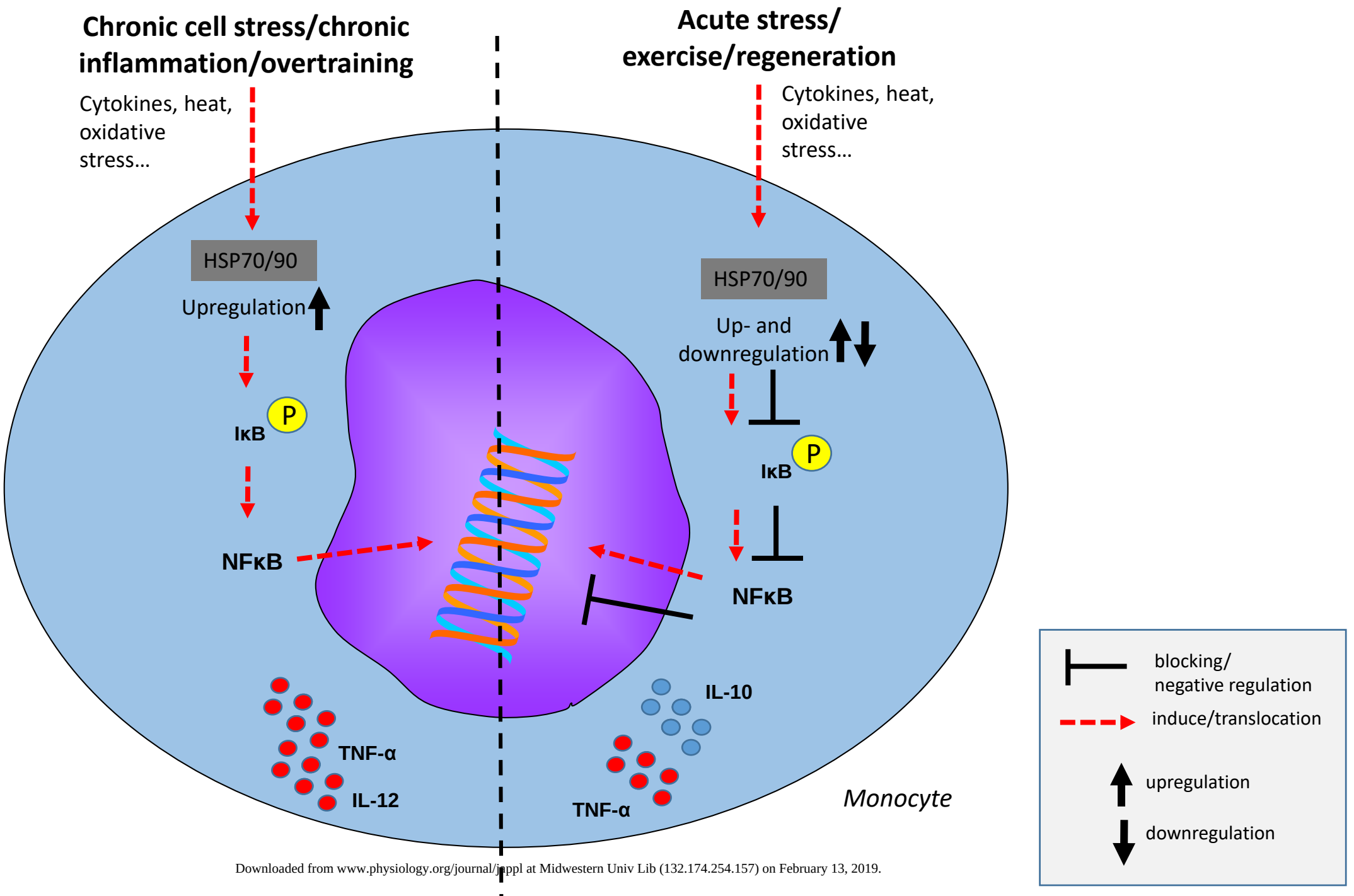
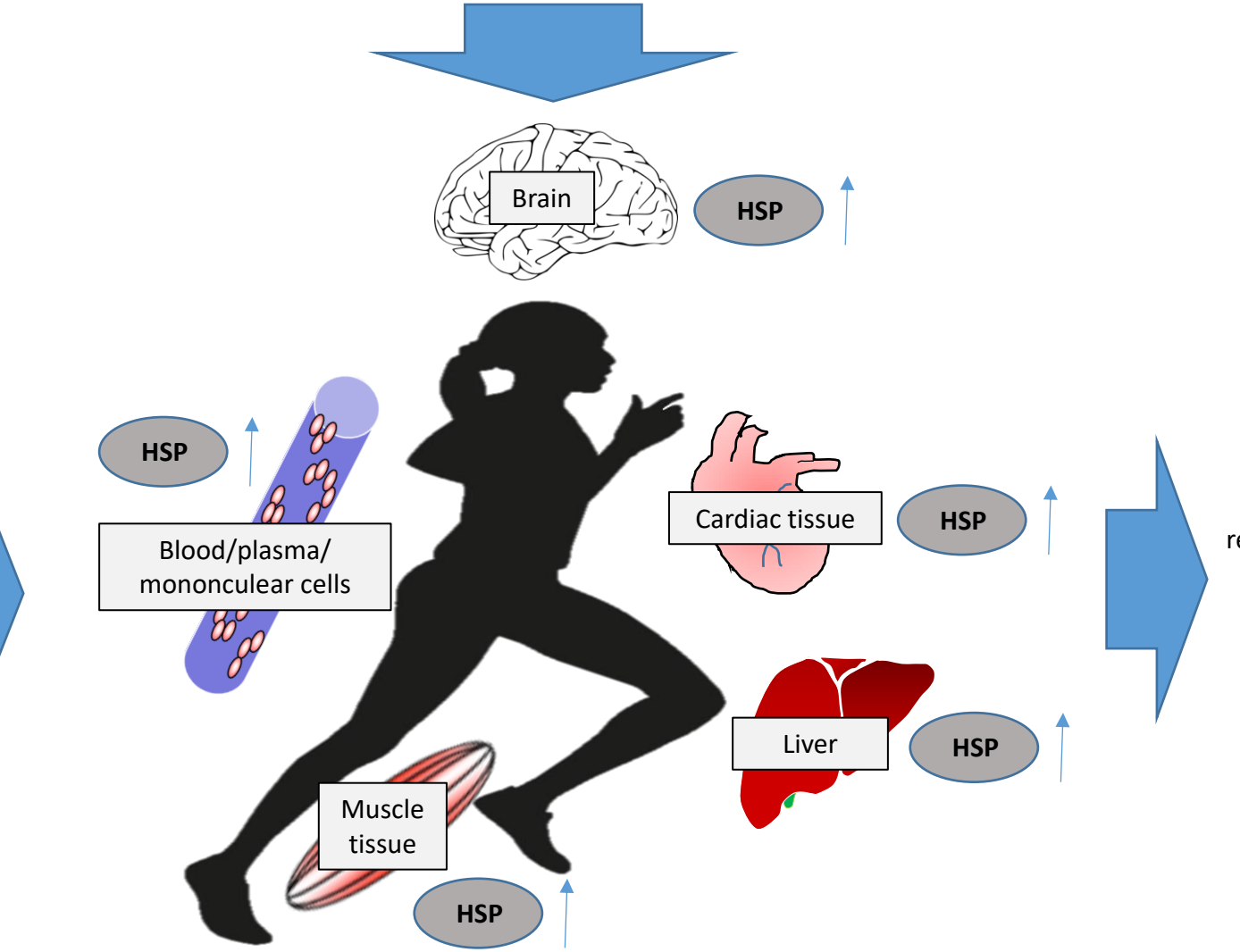




Physiological triggers:
hypoxia
oxidative stress
heat production
intracellular Ca^{2+} signaling
glycogen depletion
disturbed membrane integrity



Dependent factors:
duration,
intensity,
type/modality,
training status,
environmental factors
(temperature…)

Physiological relevance:
immune regulation
regeneration and reparation
restoring tissue integrity,
inducing adaptation
cell protection
preconditioning

Figure 3