Skeletal Muscle and Stress Proteins

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All organisms and cells respond to various stress conditions by up-regulating the expression and/or activation of a group of proteins called heat shock proteins (HSPs). Although their expression is induced by several stimuli, they are commonly recognized as HSPs due to the first experiments showing their increased transcription after application of heat shock. These proteins are molecular chaperones mainly involved in assisting protein transport and folding, assembling multimolecular complexes, and triggering protein degradation by proteasome. In addition, they play a crucial role in gene expression regulation, DNA replication, signal transduction, cell differentiation, apoptosis, cellular senescence or immortalization, and intercellular communications. Heat shock proteins are classified according to their molecular weight in super heavy, 100, 90, 70, 60, 40, and small HSPs. Although they are the most highly conserved, ubiquitous, and abundant proteins in all organisms, their cellular stress response can depend on the class and stimulus.

skeletal muscle physical exercise heat shock protein 60

1. Fiber-Types of Skeletal Muscle and Its Adaptation to Physical Exercise

Skeletal muscle is a heterogeneous tissue containing fibers with different morphological, metabolic, and functional properties. Muscle fibers are typically identified by the expression of a multigene family of Myosin Heavy Chains (MHCs). Their sequence variability has been associated with their specific structure and function, but the codified actin-based motor proteins are conserved [1][2]. In the skeletal muscle they are classified as MHC-I, MHC-IIa, MHC-IIx, and MHC-IIb [1][2][3]. The MHC-I, IIa, and IIx fiber-types are expressed to variable degrees in both small animals (mice, rats, and rabbits) and human skeletal muscle. Instead, MHC-IIb fibers are solely expressed in small animals' skeletal muscle [4]. In mammals, ''hybrid'' fibers (i.e., type I/IIa, IIa/x, IIx/b) can occur when different MHC transcripts coexist in a single fiber [5][6][7].

Although the structural and functional muscle requests define privileged associations between MHC isoforms, other common fiber-type classifications are used. One of them is related to the contractile response (or speed of contraction) distinguishing between slow or fast muscle fibers. In addition, cellular metabolism has been used as another parameter to distinguish the different types of myofibers with glycolytic metabolism and fibers with a massive mitochondrial presence and prevalent aerobic metabolism. Notably, MHC isoform expression correlates with fiber-type morphology, metabolism, and function [8][9]. In general terms, MHC-I-expressing fibers are small, rich in oxidative enzymes, slow in contraction, and have a greater resistance to fatigue, while MHC-IIb-expressing fibers are large, rich in glycolytic enzymes, and fast in contraction due to the developed sarcoplasmic reticulum that

allows the rapid release of calcium ions, and a predominantly anaerobic metabolism [10][11]. Specifically, fiber contraction correlates with myosin ATPase activity with relative velocities of I < IIa < IIx < IIb [12][13]. Furthermore, skeletal muscle can be classified as postural or non-postural according to its function and the percentage of each fiber-type [14]. Although the four main types of skeletal muscle fibers are present in different percentages among the mammal species and within the same species therein, they are represented differently in the various structures of the musculoskeletal system (Table 1) [15][16][17][18]. Moreover, basal mitochondrial content has been shown to vary between species and is fiber-type-specific in mouse, rat, and human skeletal muscles [19].

Table 1. Fiber-type distribution and mitochondrial content in different skeletal muscles.

Skeletal Muscles	Species	Gender	Main Fiber-Types %	Mitochondrial Content	References
Vastus Lateralis	Human	Male	I 49–IIa 42%	I > IIa > IIx	[14][18]
Plantaris	Rat	Male	IIx 45–IIa 21%	IIa > I > IIx > IIb	[14][18]
Plantaris	Rat	Female	IIb 46–IIx 40%		[15]
Soleus	Rat	Male	I 97%	IIa > I > IIx > IIb	[14][18]
Soleus	Rat	Female	I 99%		[16]
Gastrocnemius	Rat	Male	IIx 43–IIb 26%		[14]
Soleus	Mouse	Male	IIa 49–I 31%		[14]
Soleus	Mouse	Female	I 49–IIa 35%		[17]
EDL	Mouse	Male	IIb 63–IIx 18%		[17]
EDL	Mouse	Female	IIx 37–IIb 35%		[17]
Gastrocnemius	Mouse	Male	IIb 56–IIa 21%	IIa > IIx > I > IIb	[14][18]

EDL, extensor digitorum longus; I, type I; IIa, type IIa; IIx, type IIx; IIb, type IIb.

One of the most surprising characteristics of the myofibers of the skeletal muscle is the high degree of plasticity as an adaptive response to physiological and non-physiological requests [20]. Plasticity is the ability of a tissue to modify its composition by adapting it to changing functional needs. Repeated, prolonged, or simple changes in functional requests can both quantitatively and qualitatively modify muscle tissue. These changes can affect the myofibrillary system, the sarcoplasmic reticulum, the proteins involved in regulating the concentration of intracellular calcium as well as the enzymatic systems involved in energy metabolism. The mechanism of adaptation of the functional requests is based primarily on the transformation of the MHC content, with a shift of the MHC fibers that affects the overall speed of contraction of the muscle. However, it is also possible that an increase in the speed of the same cellular type occurs in the absence of variations of the expressed MHC isoforms [21][22]. The increased contractile activity following physical exercise activates several signal pathways that lead to

significant phenotypic changes such as MHC fiber transitions, enhanced mitochondrial biogenesis, and angiogenesis. Changes in the expression pattern of MHC isoforms and in the cross-sectional area (CSA) of the skeletal muscle cells, in response to different training protocols, are related to the changes in strength and power that the muscle undergoes [23]. For these reasons, physical exercise induces muscle hypertrophy that is followed by the upregulation of contractile elements' synthesis. Literature data have shown the hypertrophic response for all three major types of fibers (MCH-I, IIa, and IIx) following resistance training, both in young subjects and in elderly subjects [24]. However, exercise-induced hypertrophy seems to affect fast muscle fibers more than type I fibers [25]. Exercise can therefore induce changes in the expression of MHCs, thus causing a switch from type IIb to IIx and IIa and, in rare cases, also to type I. In most cases, physiological adaptations to increased activity induce a switch to a more oxidative fast phenotype [3]. Allen et al. showed a significant increase in the percentage of fibers expressing MHC-IIa and a concomitant decrease in the percentage of fibers expressing MHC-IIb in mouse fast muscles after some weeks of wheel exercise [26]. The switch in the range IIb–IIx–IIa in mouse and rat muscle or in the range IIx–IIa in human muscle likely reflects the total amount of activity [27].

Human studies have shown that strength training induces an increase in type IIa and hybrid IIa/x fibers at the expense of fast IIx fibers. At the same time, there is an increase in the CSA in all types of myofibers, indicating a hypertrophic effect [28]. In fact, Kesidis et al. showed that type IIa muscle fibers in human skeletal muscle seem to have an enzymatic profile and a rate of contraction that makes these fibers more suitable for strength performance than fibers containing the MHC I isoform [29]. On the other hand, endurance training seems to induce fast-to-slow fiber transitions (from IIx to IIa, and in rare cases type I), while the CSA values remain unchanged. The physiological advantage is the greater transduction efficiency of the mechanical energy associated with the IIa fibers compared to the IIx fibers. However, the increased proportion of type I fibers could derive from different training protocols, causing a significant turnover and regeneration of fibers, and also including a regeneration of the peripheral nerve [30]. The absence of cellular damage can be explained by the lack of the expression of embryonic myosin isoforms and supports the results of the studies in which no variations in fiber-type I following training protocols were detected [31].

2. Stress Proteins: Heat Shock Protein 60

The HSPD1 gene comprises ~17 kb with 12 exons and it is localized at chromosome locus 2q33.1. This gene encodes a protein of 573aa corresponding to a molecular weight of 61.05 kDa known as HSP60 or Hsp60, also commonly referred to as Cpn60 [32]. Hsp60 belongs to group I of chaperonins [33]. Its ATP-dependent chaperon mechanism was thoroughly investigated for the prokaryotic homolog GroEL. Three structural domains were identified for GroEL: apical, intermediate, and equatorial. To carry out its chaperoning function, GroEL needs to generate a tetradecamer complex with its co-chaperon GroES (the homolog of Hsp10) [34]. Thus, the chaperon complex is made up of GroEL, structured in two rings with seven identical subunits, and GroES, which binds to the apical domains of GroEL to close the cage [35]. The chaperon mechanism is a multistep process that involves the unfolded protein binding to GroEL apical domains. Concomitantly, ATP binds to GroEL's equatorial domain and its hydrolysis allows the conformational change (from trans to cis) of the GroEL apical and intermediate domains for

the substrate encapsulation in the central cavity of the chaperon [36]. Consecutively, the dissociation of the cis-complex and the release of the folded protein, ADP, and GroES occurs [37]. Although the chaperon mechanism of the mammalian mitochondrial Hsp60-Hsp10 complex is similar, the solid-state structure appears as a symmetrical football-shaped complex by X-ray [38]. In humans, the mitochondrial Hsp60 exists as a homo-oligomer of seven subunits in equilibrium with very minor populations of monomers and double-ring tetradecamers [39][40]. In addition, it was demonstrated that Hsp60 single ring could perform chaperonin-mediated folding activity in vivo [41].

Hsp60 is constitutively expressed under philological conditions, so much so that its knockout is incompatible with life [42][43][44]. At the same time, Hsp60 expression is related to numerous etio-pathological conditions [45]. Hsp60 is mainly localized in the mitochondria. The mitochondrial import signal (MIS) at the N-terminus drives Hsp60 from the cytoplasm to the mitochondria [46]. Nevertheless, one third of Hsp60 is localized to the extra-mitochondrial sites, such as cytosol, plasma-cell membrane, inside exosomes, extracellular space, and circulation [47][48]. Inside the mitochondria, Hsp60 guarantees the correct folding of other mitochondrial proteins [49][50] as well as its “unfoldase” activity to stabilize misfolded and aggregated proteins, making provision for the mitochondrial biogenesis and protein homeostasis [51][52]. In addition, the mitochondrial Hsp60 directs the replication and transmission of mitochondrial DNA (mtDNA) [53][54]. Otherwise, the extramitochondrial Hsp60 is involved in intracellular protein trafficking [55] and peptide-hormone signaling [56]. Interestingly, the mitochondrial and the cytosolic Hsp60 have a contradictory role in pro-apoptotic and pro-survival mechanisms [57]. Whether Hsp60 is associated with carcinogenesis, specifically with tumor cell survival and proliferation, for certain tumors is used as a good diagnostic marker [58][59][60][61]. Therefore, Hsp60 exerts divergent roles in several physiological and pathological processes, and an understanding of its structural and functional biology aspires to draw novel pathways and to develop therapeutic strategies.

3. Stress Proteins: αB-Crystallin

HSPB1, or the CRYAB gene, encodes a 175-amino acid protein with a molecular mass of ~20 kDa [62]. CRYAB is a ubiquitous sHSP with highly conserved stretch that adopts a β-sandwich, immunoglobulin-like fold called the “α-crystallin domain (ACD),” which is a characteristic hallmark of the sHSPs family [63]. The ACD region is flanked by a less conserved and flexible N-terminal domain (NTD) and a C-Terminal extension (CTE), which are variable in length and sequence except for few conserved stretches [63][64].

Based on all findings related to CRYAB missense, truncating, and frame-shift mutations, specific functional roles of these domains have been hypothesized. Indeed, mutations within ACD domain (i.e., D109H/D109A, R120G) seem to interfere with the CRYAB oligomerization processes [65][66][67], while those within the CTE domain (i.e., 464delCT, R151X, G154S, L155fs_163X, R157H) seem to compromise CRYAB chaperone function [68][69][70][71][72][73]. Finally, mutations within the NTD domain seem to prevent the building of higher order oligomeric structures [74]. Thus, oligomeric assembly and chaperone activity of CRYAB is inter-dependent on its NTD, ACD and CTE domains.

As all sHSPs do, CRYAB shares in the property to form globular oligomer structures that in mammalian cells are characterized by molecular masses ranging from 50 to about 800 kDa. This ability, together with the well-known

hetero-oligomerization property, is crucial factor in regulating the activity of this protein [75]. This hetero oligomer is probably unable to play an efficient protective role in stress conditions. However, the dissociation of the complex after-exposure to heat shock or oxidative stress suggests that it could bear new protein target recognition abilities and/or modulate those of the parental molecules [76].

Another intriguing property of sHSPs concerns its ability to be phosphorylated and therefore susceptible of control by several transduction pathways. Depending on the type and/or duration of various stimuli, the fraction of phosphorylated CRYAB ranges between 10% and 27% [77][78]. Different studies demonstrate that the phosphorylation of CRYAB shows a dual role that leads to either beneficial or deleterious outcomes depending on the extent and duration of stress and subsequent degree of phosphorylation; a phosphorylation at initial stage of stress is usually reversible and seems to provide a beneficial outcome, while prolonged stress can induce an irreversible phosphorylation which may lead to a deleterious outcome [79]. The CRYAB has three phosphorylation sites (S19, S45, and S59) at the NTD, which play a critical role in the protein functions. While the phosphorylation on S59 is mediated by p38 mitogen-activated protein kinase (p38 MAPK) and phosphorylation on S45 by the extracellular signal-regulated kinase 1/2 (ERK1/2) [78][80], the kinase responsible for phosphorylation on S19 is still unknown. Nevertheless, both unphosphorylated and phosphorylated forms of CRYAB are reported to be equally effective in preventing in vitro assembly of glial fibrillary acidic protein and vimentin in an ATP-independent manner [81]. In fact, during physiological or pathological stress both CRYAB content and phosphorylation can be modulated [82][83][84][85][86].

All aforementioned serine residues can be phosphorylated after various stimuli [78], but only a few studies have reported their contemporary involvement in muscle tissues [87][86][88][89][90]. To date, most of the available data are related to CRYAB expression and/or activation at Ser59 [82][83][91][92][93][94][95][96]. Moreover, the relationship between the phosphorylation of CRYAB and its chaperone activity is contradictory. Though in general the phosphorylation has an augmentative effect, it is possible that modulation of the activity upon phosphorylation might depend on the target protein and its interactions [97]. Further details about the phosphorylation of CRYAB in various physiological or pathological conditions can be found elsewhere [79][98].

In addition to being overexpressed in stress conditions, CRYAB shares the ability of having a tissue/cell-specific expression in the absence of stress, which can be detected in healthy adults as well as during organism development [99]. In mammalian cells, CRYAB is constitutively expressed in tissue with high rates of oxidative metabolism, including the cardiac and skeletal muscle [100]. The significance of the constitutive expression of this sHSP is probably linked to the protection of the cells against chronic stress or to a specific function in a particular tissue.

4. Hsp60 in Skeletal Muscle Fibers

The chaperoning systems that participate in controlling cellular homeostasis have been detected in skeletal muscle. Small Hsp, Hsp60, Hsp70, and Hsp90 play a significant role in muscle adaptation [101][102]. Nevertheless, Hsp60 was not deeply investigated after physical exercise, which, as we have already discussed, influences

muscle homeostasis [47]. Hsp60 and exercise correlation appears to be rational, but literature data are restricted and sometimes controversial (Table 2). Morton et al. [103] demonstrated that aerobically trained men had significantly higher resting levels of Hsp60 in the vastus lateralis muscle (high percentage of fibers I and IIa) compared to untrained subjects, suggesting Hsp60 as a molecular marker of physiological adaptation to aerobic exercise. However, it has been demonstrated that in the human vastus lateralis muscle the highest mitochondrial content is showed by type I fibers followed by type IIa > IIx (Table 1) [19]. At the same time, an acute single bout of endurance training that is considered an aerobic exercise did not increase the basal Hsp60 protein levels in the same muscle [103]. Thus, chronic training has the capacity to increase Hsp60 expression, whereas a single bout of exercise does not. Hsp60 expression is stimulated by endurance, resistance, and mixed training, but its fiber specificity is still debated. Hsp60 expression in the vastus lateralis of healthy active people with different training backgrounds was considered not to be fiber-type specific [104]. In agreement, Ogata et al. [105] and Soares Moura et al. [106] did not show significant differences in Hsp60 levels in the plantaris and gastrocnemius, both rich fiber IIx muscles, of male rats after endurance training. On the other hand, several groups, including ours, demonstrated fiber-type specificity after training in specific muscles. Mattson et al. [107] showed that female rats trained with an endurance protocol for 8 weeks displayed significantly higher levels of Hsp60 in the muscle plantaris, which is rich in fiber-type IIb [16]. No difference of Hsp60 levels was detected in the rich fiber I muscle soleus in endurance-trained rats compared to the untrained group [17][107]. Hsp60 fiber-type I specificity was reported by Samelman [108], who showed increased basal levels of Hsp60 in the soleus and not in the lateral gastrocnemius of endurance trained rats. In agreement, our group noted Hsp60 fiber-specific expression in healthy male BALB/c mice trained for 45 days on the treadmill. Specifically, higher levels of Hsp60 were observed in type I and IIa muscle fibers, while type IIx and IIb fibers showed a constitutive expression of this chaperonin [109]. Therefore, increased levels of Hsp60 were reported after six weeks of endurance training, mainly in red gastrocnemius and soleus muscles, which are particularly rich in type I and IIa fibers [109]. We also correlated this physiological adaptation to an increased expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), which triggers the mitochondrial biogenesis and thereby avoiding the cytotoxic effects [109][110][111]. Finally, increased levels of Hsp60 were observed in the soleus muscle of male mice and the Extensor Digitorum Longus (EDL) muscle of female mice after an acute single bout of endurance training [18].

Table 2. Hsp60 expression levels in different skeletal muscles after physical exercise.

Species/Strain	Gender (Age)	Skeletal Muscles	Protocol Training	Hsp60 Levels	References
Human	Male (28 ± 6 yrs)	Vastus Lateralis	Endurance	↑	[103]
Human	Male (28 ± 6 yrs)	Vastus Lateralis	Acute exercise	=	[103]
Rat/Wistar	Male (4 months)	Plantaris	Endurance	=	[105]
Rat/Wistar	Female (ns)	Plantaris	Endurance	↑	[108]
Rat/Fischer 344	Male (10 months)	Soleus	Endurance	↑	[108]
Rat/Wistar	Female (ns)	Soleus	Endurance	=	[107]

Species/Strain	Gender (Age)	Skeletal Muscles	Protocol Training	Hsp60 Levels	References
Rat/Wistar	Male (ns)	Gastrocnemius	Endurance	=	[106]
Rat/Fischer 344	Male (10 months)	Gastrocnemius	Endurance	=	[108]
Mouse/BALB/c	Male (7 weeks)	Soleus	Endurance	↑	[109]
Mouse/BALB/c	Male (12 weeks)	Soleus	Acute exercise	↑	[18]
Mouse/BALB/c	Female (12 weeks)	Soleus	Acute exercise	=	[18]
Mouse/BALB/c	Male (12 weeks)	EDL	Acute exercise	=	[18]
Mouse/BALB/c	Female (12 weeks)	EDL	Acute exercise	↑	[18]
Mouse/BALB/c	Male (7 weeks)	Gastrocnemius	Endurance	=	[109]

Hsp60, heat shock protein 60; EDL, extensor digitorum longus; arrow, increased levels of Hsp60; =, no difference; ns, not specified; yrs, years.

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