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Review

Contribution of small heat shock proteins to muscle development and function

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ABSTRACT

Investigations undertaken over the past years have led scientists to introduce the concept of protein quality control (PQC) systems, which are responsible for polypeptide processing. The PQC system monitors proteostasis and involves activity of different chaperones such as small heat shock proteins (sHSPs). These proteins act during normal conditions as housekeeping proteins regulating cellular processes, and during stress conditions. They also mediate the removal of toxic misfolded polypeptides and thereby prevent development of pathogenic states. It is postulated that sHSPs are involved in muscle development. They could act via modulation of myogenesis or by maintenance of the structural integrity of signaling complexes. Moreover, mutations in genes coding for sHSPs lead to pathological states affecting muscular tissue functioning.

This review focuses on the question how sHSPs, still relatively poorly understood proteins, contribute to the development and function of three types of muscle tissue: skeletal, cardiac and smooth.

1. Introduction

Organisms, to reduce their susceptibility to various environmental and cellular stresses, have developed so-called protein quality control (PQC) systems, in which all members of the small heat shock protein (sHSP) family take part. sHSPs are the first line of defense against misfolded polypeptides with a tendency to aggregation. Abnormalities in this defense mechanism caused, e.g. by mutations in genes encoding its components, may lead to disruption of protein folding, structure and function, and can result in diseases such as skeletal and cardiac myopathies and neurological disorders [for review see [1]].

sHSPs (HSPBs) [2] are described as molecular chaperones involved in the response to stress conditions such as heat or oxidative stress [3]. The activity of sHSPs does not require ATP, and unlike some other heat shock proteins they are unable to refold damaged proteins. Their main task is maintaining the soluble state of unfolded proteins and preventing their precipitation [3] (Fig. 1). Representatives of this family are known from all domains: Archaea [4], bacteria [5], and eukaryotes, including fungi [6], plants [7] and animals [8]. Most of the organisms have several different homologues of sHSPs: a few in bacteria and yeast [6], 10 in humans [9], 13 in zebrafish [10], and up to 15 in plants [11]. In higher eukaryotes the distribution of each sHSP is subcellular and/or tissue specific [12,13]. Mutations in sHSPs are connected with some severe pathologies such as desmin-related myopathy, distal hereditary motor neuropathy, Charcot–Marie–Tooth (CMT) disease, cataract and neurodegenerative diseases [14].

1.1. Structure and activity of sHSPs

The mass of a single sHSP molecule varies between 15 and 40 kDa. Each sHSP contains a highly conserved 80–100 amino acid α-crystalline domain (ACD), essential for its activity. There are a few atomic resolution structures obtained from whole molecules – the archeon *Methanococcus jannaschii* [4], wheat *Triticum aestivum* [7] and tape worm *Taenia saginata* [8] – and some from versions shortened on both ends containing an ACD core [15]. The ACD forms an immunoglobulin-like β sandwich fold composed of nine β strands. Two β sheets of the β sandwich are composed of respectively four (β2, β3, β8, β9) and three (β4, β5, β7) β strands. The core α-crystalline domain is flanked with longer N-terminal and shorter C-terminal regions, variable in length and amino acid composition [16]. Interactions between pockets formed in the α-crystalline

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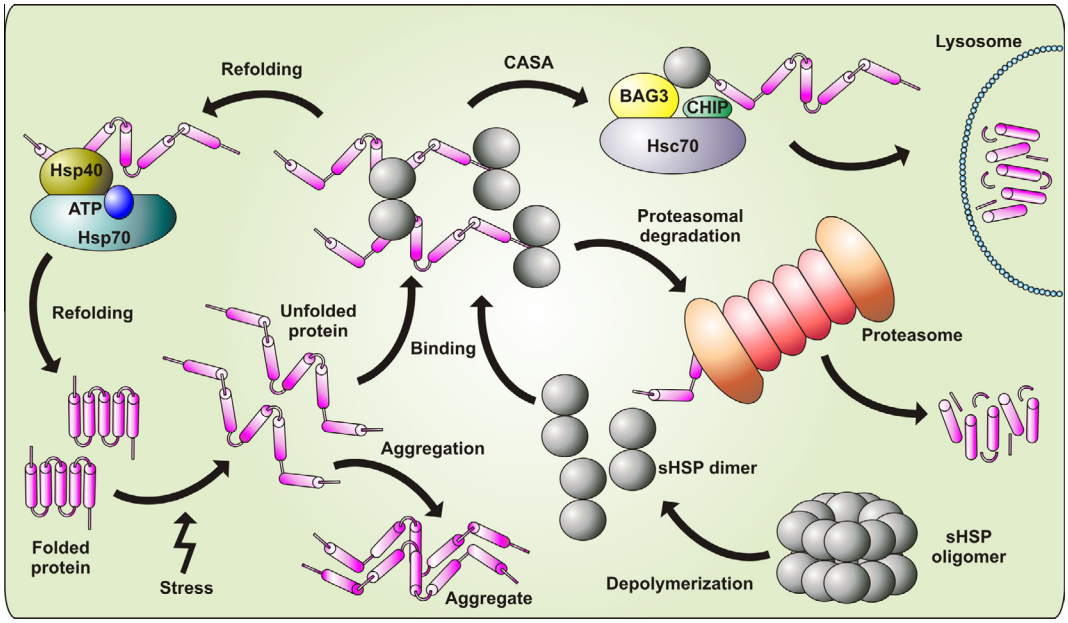

Fig. 1. Involvement of sHSPs in maintenance of cellular protein homeostasis. The main task of sHSPs is maintaining the soluble state of unfolded proteins and preventing their precipitation and formation of potentially toxic aggregates. They are part of the multicomponent machinery responsible for monitoring all steps of the protein life cycle which comprises *inter alia* synthesis, folding, aggregation, refolding and degradation. sHSPs participate, together with the Hsp70/Hsp40 machinery, in restoration of native conformation of unfolded or partially folded polypeptides, or in their proteasomal degradation when the repair of damage is impossible. Due to interactions with different partners some sHSPs direct proteins to alternative degradation pathways such as CASA (chaperone-assisted selective autophagy). See main text for a detailed description.

domain and terminal extensions play a role in the further assembly of sHSP and in the formation of clusters [17,18].

In the inactive state most sHSPs form large oligomers, assembled from both homo- and heterodimers [19]. Monomers within a dimer are connected in an antiparallel manner, by reciprocal fitting of a β6 strand into the neighboring protein molecule or by interactions between elongated β6/β7 strands [18]. The C-terminal region is rather involved in dimer formation, whereas the N-terminal region is found to stabilize highly organized oligomers [16,17]. The C-terminal end contains a conservative I/V/L-X-I/V/L sequence which is reported to fit inside the β4/β8 groove [20] and is responsible for the intrinsically disordered state of this part of the protein. Since forms of sHSPs truncated on the C-termini are connected with cataract, the accessibility of the β4/β8 groove affected by a flexible C-terminus may be significant in regulation of chaperone activity [18,21]. Dissociation of both monomers and dimers from the oligomers is required for sHSP activity, and is regulated by phosphorylation of multiple sites in the N-termini [22,23]. Additionally, the dimeric state enables creation of a hydrophobic groove between monomers, essential for binding unfolded peptides [24]. Distortions and charge shifts present in many mutated forms of sHSPs slightly alter the interfaces inside the dimer and cause closure of the groove [24].

The most prominent activity of sHSPs is binding proteins and protecting them from aggregation. Antiaggregation properties of sHSPs may inhibit aggregation of many pathogenic proteins which cause various neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), dementia with Lewy bodies or Huntington's disease (HD). Formation of filaments by both wild-type and mutated (PD) forms of alpha-synuclein can be stopped in the presence of sHSPs, especially HSPB8 [25]. There are also reports suggesting that some sHSPs enhance dissolving of senile plaques present in AD [26,27] and suppress aggregation of polyglutamate proteins by interacting with their Josephin domain, which is responsible for the initial steps of aggregation of polyQ proteins [28]. It is suggested that some of these effects can be caused by promoting autophagy and/or an inflammatory response after detection of misfolded proteins by sHSPs [29–31].

sHSPs are able to bind most unfolded proteins in a non-specific manner, preventing their aggregation; however, there are also more regular partners and a pin array assay indicated specific binding sites for different proteins. HSPB5 (a.k.a αB-crystallin) has, aside from chaperone sites, the ability to bind intermediate filaments (desmin, GFAP), actin microfilaments and some growth factors [32–34]. Also HSPB8 is noted to interact with partner protein Bag3 [35], which connects sHSP with Hsc70, directing proteins to refolding, and to autophagy or proteasomal degradation [29,30,36,37].

1.2. Interaction of sHSPs with cytoskeletal elements and other binding partners

Many representatives of the sHSP family – HSPB1 (a.k.a HSP27), HSPB4, HSPB5 and HSPB6 – have been studied in the context of their cytoskeleton interactions. Their interactions with the cytoskeleton and abilities to affect its structure and dynamics are mainly described at the level of microfilaments and intermediate filaments (IFs) [34,38–41]. Different sHSPs were noted to colocalize with various IFs such as keratin 18, GFAP or desmin [34]. All mentioned sHSPs stabilize and modulate monomer assembly/disassembly of the filaments. The character of this activity is closely connected with the phosphorylated/unphosphorylated state of sHSPs. For example, unphosphorylated HSPB1, in its large oligomeric state, is able to bind up to 30 actin monomers, whereas its phosphorylation leads to disintegration of the large complex [42]. Since HSPB5 can inhibit both aggregation and assembly of desmin, mutations in its crystalline domain that increase affinity toward IF cause aberrant desmin aggregation [43,44]. A similar mechanism can also lead to formation of vimentin aggregates in cataract originating from HSPB5 mutation [45]. Additionally, CMT disease phenotype caused by HSPB1 (S135F) mutation also corresponds to abnormal microtubule stabilization due to higher affinity of the

chaperone molecule toward client proteins [46]. On the other hand, HSPB1 in the unphosphorylated, monomeric state inhibits polymerization of actin microfilaments by capping their plus end [47], whereas phosphorylated forms are involved in filament dynamics and protect the cell from defective actin aggregates [48,49].

Most sHSPs also show antiapoptotic properties and are overexpressed in various types of cancer [50,51]. Aside from binding misfolded proteins and intermediate apoptosis prevention, some sHSPs can also directly interact with proapoptotic and antiapoptotic proteins, through specific pathways and active sites which differ among sHSPs.

HSPB1 appears to have a major role in apoptosis regulation. This protein is able to prevent apoptosis caused by heat shock, oxidative stress, ischemia or apoptosis effectors. HSPB1 stops release of Smac (small mitochondria-derived activator of caspases) to the cytoplasm, and binds already released cytochrome-c and caspase 3 [52,53]. It was also reported to indirectly inhibit translocation of Bax into the mitochondria, through PI-3 kinase activation which activates prosurvival kinase Akt and promotes its interaction with Bax [54]. HSPB1 is also implicated in increased phosphorylation and cytoplasmic localization of cyclin-dependent kinase inhibitor p21, promoting cell survival and cell cycle progression. In contrast, inactivation of HSPB1 prevents p21 translocation, stops the cell cycle and enhances cell death [55,56]. Another antiapoptotic pathway consists in the protection of eIF4E (eukaryotic translational initiation factor 4E) from its ubiquitin–proteasome degradation [57,58]. In the caspase-independent apoptosis pathway, HSPB1 prevents binding of Daxx (death domain-associated protein 6) with Ask1 (apoptosis signal regulated kinase 1) and Fas (regulator of cell death), and inhibits their proapoptotic activities [59]. HSPB1 also contributes to resistance to doxorubicin and other anticancer drugs [55]. Additionally, HSPB1 oligomer size, phosphorylated sites and subcellular localization differ depending on the character of apoptotic effectors. Etoposide and Fas antibody presence results in accumulation of medium and large oligomers, while staurosporine and cytochalasin B induce a small oligomer state [60]. HSPB1 has also neuroprotective properties derived from reducing the toxic effect of mutated SOD1 in ALS (amyotrophic lateral sclerosis) disorder [61,62].

The antiapoptotic function of HSPB5 was shown to involve inhibition of the RAS-initiated RAF/MEK/ERK signaling pathway. Studies on rabbit lens epithelial cells revealed that HSPB5 overexpression decreases the apoptosis rate by preventing RAS activation [63]. Downstream HSPB5 is able to stop Bax and Bcl-2 translocation from cytoplasm to mitochondria, as demonstrated in retinal pigment cells [64]. A similar effect was observed in myoblast cultures treated with hydrogen peroxide, where HSPB5 interacted with p53 to retain it in the cytoplasm [65]. Additionally, HSPB5 inhibits autocatalytic maturation of caspase-3 [66]. More recent studies supported this finding *in vivo*, showing antiapoptotic properties of HSPB5 in stressed retinal pigment epithelial cells [67].

HSPB8 is associated with cell proliferation and protection from apoptosis in melanoma, glioblastoma and breast cancer cells [68–70]. Depending on the cancer type the mechanisms of HSPB8 antiapoptotic activity may differ. In melanocytes overexpression of HSPB8 enhances tumor transformation during the G1 cell cycle stage. On the other hand, in glioblastoma cells HSPB8 opposes Sam68, and facilitates cell cycle progression from G1 to S by enhanced expression of cell cycle regulatory proteins such as cyclins E and A or PCNA [69]. HSPB8 is also able to activate the PI3K/Akt pathway in cardiac cells, and supports their growth and survival [71]. Yet in some other tumor tissues the level of HSPB8 is lower than in normal cells, and restoring normal concentrations by both DNA demethylation and cell transfection results in apoptosis induced in two independent pathways: caspase- and $p38^{MAPK}$-dependent [72]. Thus the role of HSPB8 cannot be simply described as pro- or antiapoptotic. It is notable that the dual nature of this protein is not an exception among sHSP family members.

2. Involvement of sHSPs in skeletal muscle

2.1. Myogenesis

Skeletal muscle myogenesis is a developmental cascade orchestrated by many various intrinsic and extrinsic factors. During the early steps of myogenesis mononucleated myogenic cells divide mitotically; they then withdraw from the cell cycle to become myoblasts, and later fuse into multinucleate myotubes that differentiate into adult muscle fibers [73].

The key step for the entry of progenitor muscle cells into the myogenic program is the induction (by signals emanating from nearby tissues, such as neural tube, the notochord, and the dorsal and lateral ectoderm) of myogenic bHLH (basic/Helix-Loop-Helix) myogenic regulatory factors (MRFs). The regulatory gene family involved includes *MyoD* [74], *Myf-5* [75], *myogenin* [76] and *MRF-4* [77]. MyoD and Myf-5 are responsible for determination of myogenic precursors whereas myogenin and MRF-4 play the crucial role in terminal differentiation of muscle fibers [78]. Besides the MyoD family of myogenic transcription factors, members of the MEF2 (Myocyte Enhancer Factor 2) family of transcription factors play an important role in muscle gene activation. MEF2 proteins are MADS-box-containing transcription factors that bind as homo- or heterodimers to a conserved A/T rich DNA sequence (MEF2 site) present in the regulatory regions of many muscle-specific genes [79]. MEF2 and bHLH myogenic transcription factors cooperate synergistically to stimulate muscle-specific genes containing both E-boxes and MEF2 sites [for review see: [80]].

An important aspect of muscle differentiation is generation of multinucleated muscle fibers through fusion of mononucleated myoblasts. Several studies have shown that sHSPs are expressed during the key steps of muscle differentiation. Many researchers have turned to mouse C2C12 myoblasts, used as a model for myogenesis, to elucidate the role of sHSPs during muscle differentiation. It has been reported that an MRF-binding site exists in the gene (CRYAB)encoding HSPB5. It was also shown that in C2C12 myoblasts MyoD and myogenin can activate the HSPB5 enhancer in muscle by interaction with the MRF site [81]. The studies conducted by Ito et al. [82] revealed accumulation of HSPB1 and HSPB5 during differentiation of C2C12 cells into myotubes, a process regulated by protein kinase cascades. During C2C12 differentiation HSPB5 modulates MyoD activity by reduction of its synthesis and increasing its degradation, thus leading to delayed muscle differentiation [83]. *In vivo* studies demonstrated that during mouse myogenesis HSPB5 mRNA shows early expression in myotomes and developing skeletal muscles [84]. These data strongly suggest an important role of HSPB5 for proper myogenesis. It was also revealed that *HSPB1* is a target gene for transcription of MEF2 factors regulating advanced stages of myogenesis [85]. The authors assume that MEF2 factors, in conjunction with MyoD and other myogenic regulatory factors, may be involved in up-regulation of HSPB1 in differentiated skeletal muscles. Studies carried out by Sugiyama et al. [86] have demonstrated that skeletal muscle develops two chaperone systems which work independently in muscle maintenance and differentiation. One system includes HSPB2 and HSPB3, and the other HSPB5, HSPB1 and HSPB6. The expressions of HSPB2 and HSPB3 was observed during muscle differentiation under control

of MyoD, suggesting that they represent an additional system tightly regulated by a myogenic program, closely related to muscle differentiation. HSPB1 and HSPB5 make a myotube-specific association with actin microfilaments, which confirms their cytoprotective role [86]. It is also worth noting that in myoblasts HSPB1 was not observed, suggesting the possible involvement of these sHSPs in initial organization of myofibril assembly in myotubes. In mature muscles HSPB2 did not show a similar localization on actin bundles, although it localized on the Z-line similarly to HSPB5. In adult skeletal muscles HSPB5 was expressed in slow and fast muscles and localized in Z-bands [87].

The involvement of sHSPs in myogenesis (as well as in cardiogenesis, see Section 3.1: "Cardiogenesis") was also investigated in another model organism – *Danio rerio* (zebrafish) using "knockdown" of HSPB1 with morpholino antisense oligonucleotides in developing zebrafish embryos. Initially it was thought that in zebrafish depletion of this protein does not have an effect on skeletal or cardiac muscle morphology and functioning [88]. However, detailed analysis of morphants revealed that HSPB1 takes part in regulation of craniofacial muscle development [89]. Its depletion influences optimal growth of craniofacial myocytes rather than determination or proliferation of myogenic precursors. This observation suggests that zebrafish HSPB1 may not be involved in morphogenesis of skeletal and cardiac muscles or in myofilament organization, and its physiological role could be rather connected with protection of myocytes against mechanical or oxidative stress [90]. Similar results were obtained for a mouse model in which suppression of HSPB1 expression also did not cause phenotype changes [85]. This phenomenon was explained by a compensation effect which may be elicited by other sHSPs expressed in cardiac and skeletal muscle, e.g. HSPB2, HSPB5 and HSPB8. To examine this assumption double knockout experiments were undertaken. For example, in *Hspb5*/*Hspb2* knockout mice Brady et al. [91] observed severe muscle cell abnormalities. Loss of HSPB5 and HSPB2 functions leads to degeneration of some skeletal muscles, especially tongue and soleus muscles [91]. These findings suggest that sHSPs may be specific myofibril-stabilizing proteins. In the case of cardiogenesis, experiments carried out on double knockout mouse for *Hspb2* and *Hspb5* suggested that these protein are dispensable for the development of mouse myocardium [91,92].

2.2. Multiple functions of sHSPs in muscles

Skeletal muscle is a multinucleated, highly specialized tissue made up of over ten thousand different kinds of proteins. The dominant proteins of muscle fiber sarcoplasm are actin and myosin. Contractile proteins present in the myofibers are highly organized in a repetitive manner and assembled into sarcomeric units. Due to this contractile units muscles are able to accomplish their main task, which is putting the body in motion. On the other hand, sarcomeres are exposed to mechanical and metabolic damage. To minimize undesirable side effects in the form of protein degradation and/or accumulation of unfolded or toxic polypeptides, organisms have developed an evolutionarily conserved protection mechanism which is part of the PQC system [93]. Members of this system – sHSPs – have been shown to be of central importance in skeletal muscle development and differentiation, and are of potential significance in human diseases. Many different lines of evidence suggest that sHSPs are highly expressed in skeletal muscles and protect them from heat and from oxidative and mechanical stresses, particularly during exercise [94–96].

Seven members of the small heat shock proteins can be found in skeletal muscle: HSPB1, HSPB2, HSPB3, HSPB5, HSPB6, HSPB7 and HSPB8. To determine whether sHSPs protect skeletal muscle cells against oxidative stress, Escobedo et al. [96] used two models: differentiation-induced increases in HSPB1 and overexpression of HSBP1 in stably transfected myoblasts. The authors showed that HSPB1 protects skeletal muscle cells from reactive oxygen species (ROS)-induced damage by increasing the glutathione (GSH) level. It has been shown that an increased level of HSPB1 is associated with an increased level of GSH and decreased hydrogen peroxide-mediated cell damage as well as protein oxidation. These findings indicate that HSPB1 protects skeletal myoblasts against oxidative stress and may play a key role in regulating the GSH system and resistance to ROS in skeletal muscle cells.

It has been demonstrated that exposure of samples of rat diaphragm to heat stress results in the redistribution of HSPB6, HSPB5 and HSPB1 from the cytoplasm to insoluble fractions (a characteristic response of sHSPs to heat stress) [94]. In C2C12 undifferentiated mouse myoblasts and differentiated myotubes upon heat stress, HSPB5 and HSPB1 translocate from the cytoplasm into the nucleus and colocalize significantly with the intranuclear lamin A/C speckles in myoblasts. The authors believe that sHSPs may play a crucial role in formation and/or stabilization of the dynamics of lamin A/C architecture. Interestingly, this translocation of HSPB5 and HSPB1 into the nucleus appears to be differentiation stage-specific, implying its functional significance [97].

The engagement of sHSPs in stabilization of sarcomeric units was also investigated in an invertebrate (*Drosophila melanogaster*). During muscle contraction some proteins such as filamin undergo reversible unfolding and refolding. These periodic conformational changes make it susceptible to malfunction, which in consequence may lead to formation of toxic aggregates and disruption of myofibrillar structure. To prevent unfavorable accumulation, stress-exposed protein is coupled with a complex formed by, *inter alia*, co-chaperone BAG3 (Starvin in *D. melanogaster*), chaperone Hsc70 and HSPB8 (Dm-HSP67Bc in *D. melanogaster*) and HSPB6 [98]. Members of the complex mentioned above (e.g. HSPB8) localize in the Z band in muscle tissue, which implies their involvement in Z disk maintenance [98,99].

As stated above, sHSPs have a general function in skeletal muscles. Many different lines of evidence indicate that mutations in genes encoding sHSPs are linked to several inherited muscle defects. Numerous pathologic states of muscles are caused by mutations of the CRYAB gene. HSPB5 in normal conditions serves the role of chaperone protein in muscles, stabilizing desmin filaments, preventing their aggregation and protecting them from tension-related damage. Mutations of CRYAB genes lead to a series of muscle pathologies including one third of cases of desmin-related myopathy (DRM) (see Section 5: "Pathological aspects of sHSPs").

2.3. sHSPs protect muscles during exercise

A body of evidence has revealed that sHSPs play an important role as cytoskeleton-protective proteins under eccentric exercise (contraction with active muscle lengthening). Studies of Paulsen's group [100] showed that a single bout of unaccustomed maximal eccentric exercise stimulates acute HSPB1 and HSPB5 translocation to myofibrillar structures during exercise, with a subsequent increase in mRNA and protein levels for HSPB1 and HSPB5. Of interest, muscle lengthening contractions lead to loss of Z-disk and membrane scaffolding proteins and concomitant translocation of HSPB1 and HSPB5 to the Z-disk. Lengthening contraction-induced translocation of HSPB1 and HSPB5 was associated with phosphorylation of these sHSPs, which may promote their protective activity. This observation confirms that sHSPs may help to stabilize skeletal muscle cells and limit their cytoskeletal disruption in muscle cells through the repair of structures damaged during exercise, which may also generate ROS that can unfavorably affect cellular components [101].

During intensive muscle activity such as exercise, muscle fiber injury may occur as a consequence of the significant increase in

temperature. Therefore the involvement of some sHSPs in myofibrillogenesis and maintenance of cytoskeletal organization in conditions of hyperthermia have also been postulated. For example, HSPB5 was shown to prevent thermal unfolding and aggregation of myosin II, which allows maintenance of myosin enzymatic properties and thereby muscle contractile activity [102].

3. sHSPs in cardiac muscle

3.1. Cardiogenesis

Formation of the heart starts during gastrulation, when heart mesoderm is specified. Highly regulated migration of these precursors toward more anterior regions within the embryo occurs during neurulation. As development proceeds, cardiac precursors undergo a second migration event which comprises ventral movement toward the anterior ventral midline, where formation of the heart tube (connected with fusion and proliferation processes) occurs [103–106].

Early expression of some sHSPs (especially orthologues of HSPB1, HSPB8, HSPB5, but also HSPB6 and HSPB2) during heart formation has been reported in a wide range of animal species such as *D. melanogaster* [107], sea squirt [108], zebrafish [10,90], mouse [109], pig [110] and human [111,112]. In *D. melanogaster*, embryonic heart, which forms a contractile tube, is built of 104 cardiac cells in which expression of two sHSPs (Hsp22 and Hsp26) was confirmed. Therefore a significant role of these proteins in cardiogenesis was postulated. This phenomenon has been investigated in different animal models including zebrafish [88] and *Xenopus laevis* [113]. The expression pattern of HSPB1 is very similar in *X. laevis* and zebrafish [114,115]. In both cases its production starts in the whole embryo during the gastrulation stage; as development proceeds it is initially restricted to somites, and then expands to the entire myotome, as well as jaw and body wall muscles and heart during the tadpole stage.

In *X. laevis* involvement of HSPB1 in cardiogenesis has been proven. The role of this protein is connected with the fusion of cardiac progenitors. The presence of HSPB1 enables proper heart development, whereas its depletion leads to cardiac malformation manifesting as cardia bifida (characterized by the presence of two unfused or partially fused contractile hearts) [113]. At the subcellular level lack of HSPB1 results in perturbation of the actin filament network and myofibril disorganization. Although involvement of this protein in functional heart formation is confirmed, its lack does not affect cardiac differentiation. In the light of these data it seems plausible that HSPB1 involvement in cardiogenesis is connected with cell motility and/or adhesion [113]. However, there is some discrepancy between functions of this chaperone in *X. laevis* and other systems: *D. rerio* and *D. melanogaster*. Data obtained from these two model organisms led to the conclusion that HSPB1 is dispensable for morphogenesis but is involved in the maintenance of muscle integrity and in the stress response (see Section 2.1: "Myogenesis"). In zebrafish its orthologue could play a role in long-term maintenance of heart and muscle tissues or, as in *D. melanogaster*, could act as a chaperone involved in protection against starvation-induced stress [88,116].

3.2. Cytoskeleton dynamics

Protection of cell function during stress is connected with the ability of sHSPs to interact with cytoskeletal elements [117]. For instance, HSPB1 interacts with muscle-specific structural proteins and protects them, particularly in response to different stress agents or injury mechanisms including heat shock [118], dilated cardiomyopathy [111] prolonged exercise [100,101] and I/R (ischemia/reperfusion) injury [119].

With regard to the maintenance of cytoskeleton integrity, which is necessary for cell survival, HSPB5 seems to be crucial, as was indicated in *in vitro* and *in vivo* studies. It has been shown that HSPB5 stabilizes not only IFs but also actin filaments as well as the myofibrillar protein titin. Interactions of HSPB5 with cytoskeletal elements occur under both normal and stress conditions. Detailed analysis revealed that the binding affinity of HSPB5 to those proteins increases after heat shock. Experiments on rat cardiomyoblasts have indicated that HSPB5 binds directly to actin filaments and prevents their heat-induced as well as cytochalasin B-induced disorganization. Furthermore, because this association was abolished in the presence of MAPK inhibitors, it can be concluded that it depends on the HSPB5 phosphorylation state [48,120,121]. These mutual dependencies may be a result of a cascade in which a stressor induces disruption of the cytoskeleton, which activates p38 MAPK and in turn leads to HSPB5 phosphorylation, which then triggers its protective function towards the cytoskeleton [122]. With regard to the polymerization-interfering agents, similar effects were obtained for nocodazole-induced microtubule disassembly, which was inhibited in the presence of HSPB5 [123]. Other agents, such as oxidative stress, which have an effect on heart cells, also involve HSPB5 action. For example, translocation of the cytosolic fraction of HSPB5 to the Z-disk was observed in cardiac tissue as a result of ischemia, which suggests that HSPB5 takes part in myocardium protection [124].

HSPB1 is associated with Z-disk maintenance. Localization of HSPB1 in resting length myofibrils correlates with regions adjacent to the Z-line, which may suggest its interaction with proteins that are components of this structure. However, analysis of HSPB1 distribution in stretched myofibrils reveals that its localization does not depend on desmin, α-actinin, myosin, or filamentous actin, but is connected with titin filaments [90]. It is postulated that interaction of HSPB1 with titin, a protein critical to the maintenance of myofibril structure, or titin-associated proteins such as DRAL/FHL2 (down-regulated in rhabdomyosarcoma LIM domain protein/four-and-a-half-LIM domain protein 2), cardiac ankyrin repeat protein, and ubiquitously expressed calpain may play a crucial role in its muscle protection activity [90]. The putative interaction between HSPB1 and calpain could prevent overactivation of calpain proteolytic activity, which is a consequence of a massive calcium influx that occurs after muscle injury [90,125]. This putative interaction could also shed some light on theantiapoptotic properties of HSPB1, since participation of calpain in necrosis and apoptosis in various cell types has been proven [126].

A phenomenon strictly associated with heart functioning in which cytoskeletal rearrangement takes place is tachycardia remodeling (TR). TR is an event preceding atrial fibrillation in which a pivotal role is played by RhoA GTPase (Ras homolog gene family, member A), a small protein that regulates the actin cytoskeleton in the formation of stress fibers. At the cellular level tachycardia remodeling is connected with electrical, structural and contractility changes of the atrial myocytes as a consequence of RhoA GTPase activation [127]. It is postulated that members of the sHSP family such as HSPB1, HSPB6, HSPB7 and HSPB8 are able to modulate RhoA GTPase functioning and thereby tachycardia remodeling [128]. Their activity has a protective effect and prevents formation of F-actin stress fibers, although the way of action differs between sHSP family members. HSPB1, HSPB6 and HSPB7 directly prevent stress fiber formation, whereas HSPB8 action is indirect via inhibition of RhoA GTPase [128]. It is suggested that the unique ability of HSPB8 to inhibit stress fiber formation may be connected with its function in autophagy activation, which in turn acts as a trigger in RhoA pathway initiation [29,128].

3.3. sHSPs in response to cardiac stress (I/R)

The most abundant organelles in cardiomyocytes are mitochondria, which is the consequence of the high energetic demand of these contractile cells. The numerous mitochondria contribute to formation of significant amounts of ROS, which are capable of disturbing *inter alia* myofibrillar proteins. That implies the necessity for maintenance of redox balance in cardiomyocyte, a process in which sHSPs take part [129]. Participation of sHSPs in maintaining redox homeostasis is mostly connected with modulation of GSH level and increase of its expression level.

The expression of mammalian HSPB1 is augmented during postnatal development, which is probably connected with the increase of cardiac activity that generates ROS-induced oxidative stress [112]. Elevated levels of HSPB1 and HSPB5 were observed in hearts of mice and rats aged from 3 to 6 months [130,131] and in hearts of PIT1-deficient mice, whose lifespan is extended in comparison to wild type mice [132]. This result may suggest involvement of sHSPs in proper function of this contractile organ.

Engagement of HSPB1 and HSPB5 in cytoprotection against oxidative stress is connected with their ability to intensify the activity of enzymes such as glucose 6-phosphate dehydrogenase (G6PD), responsible for regulation of GSH level, which is known to protect cells from I/R-induced damage [133]. This is in agreement with experiments on depletion of heat shock transcription factor 1 (HSF1) in cardiac cells, which leads to downregulation of HSPB1 and HSPB5 and reduction of G6PD activity [134]. Conversely, high overexpression of HSPB1 in heart results in elevation of GSH level and in reduction of ROS production [135]. However, HSPB1 depletion does not cause expected effects such as changes in GSH and mRNA levels for several relevant sHSPs [85].

At least two sHSP family members – HSPB1 and HSPB5 – are implicated in reductive stress, which is underlain by impairment of the activity of enzymes implicated in the production and recycling of GSH. There is evidence that HSPB1 in cells plays a role as an antioxidant. An increased level of HSPB1 causes reductive stress, which leads to cardiac hypertrophy and cardiomyopathy. In cardiomyocytes reductive stress-induced disturbances are manifested by an increased ratio of reduced GSH/oxidized GSH and a decreased level of ROS, and are connected with upregulation of glutathione peroxidase 1 [135], while HSBP5 in its mutant form (R120G) contributes to the development of a multisystem protein aggregation disease with cardiomyopathy syndrome. This is also a consequence of reductive stress in the myocardium, in which an increased ratio of reduced GSH/oxidized GSH as well as mutant protein aggregation was observed. This disruption is strictly related to intensification of G6PD, glutathione reductase, and glutathione peroxidase expression [130].

Two pathological phenomena closely connected with redox imbalance are myocardium ischemia and reperfusion (I/R). These processes are closely interrelated. Ischemia is induced by an insufficient supply of blood to the heart, which obstructs delivery of oxygen and nutrients. Reperfusion in turn is the consequence of the restoration of blood flow to cardiac cells that have previously experienced deficient blood circulation. In the presence of an excessive amount of oxygen cardiomyocytes are exposed to increased ROS production, which may lead to oxidative stress-inducible tissue damage and can induce necrosis or apoptosis. sHSPs seem to be the first line of defense against I/R insult. Many different studies have proven that their overexpression protects the cell against I/R damage, as was confirmed for HSPB1, HSPB5, HSPB6 and HSPB8 [136–140]. The increased level of both HSPB1 and HSPB5 guards adult cardiomyocytes against ischemic injury. Moreover, HSPB5, in contrast to HSPB1, has a similar effect on neonatal cardiac cells subjected to simulated ischemic stress [136]. These data indicate that outcomes of I/R insult as well as protective abilities of different sHSPs are dependent on the age of the organism. The protective effects of sHSPs against I/R injury depend on their posttranslational modifications, such as phosphorylation. For instance, cardioprotective activity of overexpressed HSPB1 is even more effective when accompanied by mutations of serine residues that prevents its phosphorylation [137] whereas overexpression of HSPB6 without a specific phosphorylation site (S16) has a dramatic opposite effect [138]. Involvement of HSPB6 in cardioprotective mechanisms seems to be bipolar. On the one hand, HSPB6 attenuates cell death by interacting with Bax and thereby increasing the Bcl2/Bax ratio. On the other hand, inhibition of HSPB6 phosphorylation (S16) contributes to increased cell mortality by activation of stress-induced autophagy [141]. The significance of serine 16 in the cardioprotective activity of HSPB6 has been confirmed by identification of a mutation (P20L substitution) in its human ortholog that causes reduction of S16 phosphorylation, which in turn leads to abolition of the ability of this chaperone to protect cells against stress [142]. Overexpression of HSPB6 in the heart does not influence cardiac morphology but has an impact on its contractile function, which it intensifies due to inhibition of type 1 protein phosphatase (PP1), thereby maintaining phospholamban (PBN), an inhibitor of cardiac muscle sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), in the phosphorylated state. The phosphorylated PBN is not able to fulfill its inhibitory function, thereby allowing muscle contraction [143].

Changes in myocardial distribution of different sHSPs were observed shortly after ischemic stress exposure. [144,145]. Five of the sHSPs – HSPB5, HSPB2, HSPB1, HSPB6 and HSPB7 – relocate from the cytoplasm to the Z and/or I line regions of myofibrils, which may be stabilized by them. The translocation may also protect protein components of Z and/or I lines from unfolding or aggregation during stress conditions. HSPB2 and HSPB6 bind to actin-associated proteins, whereas HSPB5 interacts mainly with titin and desmin [146]. In ischemic cardiac cells HSPB5 is located at the Z lines present within the I line, whereas in skeletal muscles this protein is distributed throughout the entire I band. Experiments carried out on HSPB5/HSPB2-deficient hearts show an increased I/R insult with a less efficient recovery after ischemia [92,145]. These studies suggest involvement of these two sHSPs in maintaining cardiac muscle elasticity, functional recovery and protection from I/R-induced necrosis and apoptosis. Their role in the contraction process may be excluded, since the morphological and functional properties of HSPB5/HSPB2 deficient hearts were similar to WT hearts under non-stressful conditions [92,145]. Moreover, double knockout cardiomyocytes exhibit increased mitochondrial permeability transition and mitochondrial calcium uptake [147], and reduced level of GSH after ischemic stress [92]. However, since other researchers obtained contradictory data on the significance of these two sHSPs for recovery after I/R stress, this issue needs further investigation [148].

Ischemic stress induces translocation of sHSPs in cardiac cells not only to myofibrillar structures but also to other cellular components such as mitochondria. This concerns HSPB2 [149] and HSPB5 [150]. Association of HSPB5 with the outer mitochondrial membrane depends on its phosphorylation via p38 MAP, and contributes to modulation of mitochondrial damage upon reperfusion [150]. Other studies have brought further information about the distinct roles of HSPB5 and HSPB2 in cardiac cells. The first one seems to be responsible for diastolic performance and structural stability, the second for systolic performance and cardiac energetics [151].

Ischemic stress leads to upregulation of HSPB8 expression [139,152]. Experiments carried out on transgenic mice with specifically upregulated HSPB8 levels have indicated that HSPB8 is involved in cardioprotection via a nitric oxide (NO)-dependent mechanism in the context of ischemia [153]. Protective activity

of HSPB8 is similar to ischemic preconditioning (IPC), which involves activation of cell survival pathways, upregulation of heat shock proteins, inhibition of apoptosis, and metabolic adaptation [71,140]. The upregulation of HSPB8 is correlated with increased expression of the inducible isoform of nitric oxide synthase (iNOS) [140,153]. iNOS plays a role in NO-mediated adaptation in mitochondrial respiratory function, by reduction of ROS production via interaction with components of the electron transport chain. iNOS participation in mitochondrial activity inhibits proapoptotic events [154,155].

Some members of the sHSP family are also involved in the response to the oxidative stress that is an effect of anticancer drug treatment. For example, oxidative stress effects manifested by the increase of phosphorylated HSPB1 level were induced in mouse heart by chemotherapeutic drugs such as doxorubicin [156]. Accumulation of HSPB1 in the responses to drug-induced oxidative stress depends on HSF1 and leads to the loss of cardiomyocytes via activation of the proapoptotic protein Bax. Accumulation of another sHSP – HSPB6 – in mouse cardiomyocytes was also observed in the response of doxorubicin-induced oxidative stress [157].

3.4. HSPBs during cardiac hypertrophy

Cardiac hypertrophy is an abnormal enlargement of the heart muscle associated with an increased cardiomyocyte volume in response to pressure and volume overload. Cardiac hypertrophy may occur as a consequence of overexpression of some sHSPs such as HSPB1 [158], HSPB6 [159] and HSPB8 [152]. Mechanisms underlying the cardiac hypertrophy are linked to regulation of the post-translational modification of sHSP. This was recently confirmed in the case of HSPB6, whose protective abilities depend on its phosphorylation on Ser16 by PKA (protein kinase A). Phosphorylation of HSPB6 is inhibited by its direct interaction with cAMP phosphodiesterase-4 (PDE4). Attenuation of HSPB6 phosphorylation by PKA due to β-adrenergic stimulation is achieved by inhibition of its binding to PDE4. Phosphorylated HSPB6 protects against cardiac hypertrophy [159].

HSPB8 was also found in hypertrophic heart. Increase of its level in hearts of transgenic mice leads to cardiac hypertrophy proportionally to the level of its overexpression. An elevated level of HSPB8 stimulates the AKT pathway [152]. Depletion of HSPB8 in mice does not cause any significant changes in embryos developing under normal conditions and does not influence their viability [160]. Moreover, mitochondrial activity seems not to be affected by depletion or overexpression of HSPB8 in the absence of stressful agents [160]. However, HSPB8-knockout mice under stress conditions such as pressure overload show different abnormalities including hypertrophy, ventricular dilation, impaired contractile function, increased cardiomyocyte length and accumulation of interstitial collagen, faster transition into heart failure, and increased mortality. The conducted study suggests HSPB8 involvement in the mechanism of cardiac response to overload via modulation of nuclear and mitochondrial STAT3 (Signal transducer and activator of transcription 3) activity. STAT3 is a stress-inducible transcription factor responsible for cardiac cell survival [160].

The induction of myocardial hypertrophy depends on mechanotransduction involving integrins – proteins that mediate interactions with the external milieu and are reputed to be receptors of mechanical stress [161]. Integrin-mediated signal transduction involves the phosphorylation signaling cascade in which different proteins such as Focal adhesion kinase (FAK), c-Src tyrosine kinase, growth factor receptor-bound protein 2 (Grb2) and Son of Sevenless (SOS) take part [162]. This signaling induces the Ras/ERK pathway responsible *inter alia* for growth regulation. At least some of these proteins such as c-Src regulate myocardial hypertrophic growth by their association with cytoskeletal structures [162].

Another signaling protein that promotes cardiac hypertrophy is p38 mitogen-activated protein kinase (MAPK) [163]. p38 MAPK is also responsible for modulation of HSPB1 function such as regulation of the organization of the actin cytoskeleton under stress conditions [117,164]. Due to the ability to modulate the dynamics of the actin cytoskeleton, phosphorylated HSPB1 can play a role in plasma membrane-associated signal transduction. Moreover, as a result of activation of p38 MAPK, the phosphorylated HSPB1 is translocated from the cytosol to the Z-disks of myofilaments, where it takes part in protection of structural proteins such as desmin [165].

Integrin signaling, p38 MAPK, HSPB1 and cytoskeleton are involved in the hypertrophic growth induced by mechanical stress, e.g. stretch [161]. The opposite effect, i.e. atrophy, also requires engagement of the integrin pathway, HSPB1 and its upstream regulator p38 MAPK. Cardiac atrophy, which is a manifestation of adaptation to reduced myocardial load and work, occurs in response to different stress agents such as microgravity induced by hindlimb unloading [166,167].

4. sHSPs in smooth muscle

4.1. Functions of sHSPs in smooth muscles

The sHSPs HSPB1, HSPB6 and HSPB5 are chaperone proteins intensively expressed in smooth muscles. They play a significant role in the modulation of muscle contraction, cell migration and cell survival [168]. They have been found in arterial [169], airway [170], venous [171], bladder and uterine [172,173] smooth muscles. As discussed above, numerous cellular processes can be regulated by sHSPs among which binding of misfolded proteins, maintenance of cellular redox balance and actin polymerization should be mentioned. These processes influence smooth muscle proliferation, cell migration, cell survival, muscle contraction and synthesis of signaling proteins. A better understanding of sHSP functions may allow one to explain mechanisms of smooth muscle pathologies and elaborate appropriate medical treatment focused on sHSPs as potential markers or drug targets. For example, the secreted HSPB1 could be a useful marker of inflammation during atherogenesis. The phosphorylation of HSPB6, which relaxes smooth muscle, might prove to be highly relevant to treatment of hypertension, vasospasm, asthma, premature labor and overactive bladder. The phosphorylated form of HSPB6 is implicated in smooth muscle relaxation and is one of the most important phosphoproteins involved in this process [174]. Because sHSPs can also modulate smooth muscle proliferation and cell migration, they may be targets for increasing effective, novel treatments of clinical problems arising from remodeling of smooth muscle in vascular, respiratory and urogenital systems.

Numerous studies have indicated that the expression of sHSPs is very varied and depends on the type of smooth muscle and their location. HSPB1 expression is very dynamic and can increase in response to physical and chemical stressors including heat, mechanical stress, oxidative stress and proinflammatory mediators [175–177]. Similarly, HSPB6 expression in smooth muscles is also variable and depends on various conditions [178]. For example, in some vascular tissues, airway smooth muscle, and uterine smooth muscles HSPB6 is expressed constitutively [169,178,179], whereas in other tissues the expression of HSPB6 is at a low level [172].

4.2. HSPB1 in smooth muscle contraction

HSPB1 is a significant protein involved in smooth muscle contraction and migration [180]. It is commonly known that cell proliferation depends on actin filament remodeling. Moreover, cell survival is closely dependent on the integrity of the actin

cytoskeleton. As mentioned in previous sections, HSPB1 is involved in the regulation of cytoskeletal dynamics, chiefly cell cytoskeletal microfilaments. The examination of the involvement of HSPB1 in smooth muscle contraction has indicated that this process is dependent on MAP kinases and is closely connected to phosphorylation of the smooth muscle protein caldesmon. Caldesmon is responsible for the regulation of smooth muscle as well as non-muscle contraction, and is a substrate for ERK and p38 MAP kinases [181,182]. This protein, in cooperation with HSPB1 among others, is able to modulate actin–myosin interaction and thereby influences muscle contraction. After phosphorylation both proteins form a complex in which phosphocaldesmon undergoes conformational change resulting in its dissociation from tropomyosin. This leads to the sliding of tropomyosin on actin and eventually to smooth muscle contraction [182].

Actin is particularly responsible for the maintenance of cell shape. This function depends on actin polymerization, which must be orchestrated both temporally and spatially [183]. Several studies have shown that overexpression of HSPB1 increases the stability of actin microfilaments during exposure to different stress agents such as hyperthermia, redox status and cytochalasin D [184]. Under stress conditions, the integrity of F-actin is disrupted by uncontrolled rupture and aggregation of filaments, which demolishes cell morphology. It is suggested that HSPB1 interacts with F-actin during heat stress. This interaction may be an adaptive response to changes in the cellular environment, in order to stabilize the structure and prevent cytoskeleton disaggregation also in smooth muscle cells. The exact mechanism by which HSPB1 stabilizes F-actin is poorly understood.

An interaction of HSPB1 and HSPB5 with various IFs has also been reported [34]. It is proposed that these interactions could involve the connections between filaments in cellular networks, an event that might be important for the survival of cells. Evidence also suggests that HSPB1 co- localizes with tubulin/microtubules, although the significance of this observation is not fully understood [185].

4.3. HSPB1 in cell migration

Smooth muscles are exposed to numerous growth factors and proinflammatory cytokines that lead to abnormalities such as atherosclerosis, angiogenesis, and smooth muscle hypertrophy. Cell migration is another function that depends mainly on the conversion of G-actin to F-actin in smooth muscles. The common feature of these pathological changes is the migration of smooth muscle cells. The extracellular signaling pathways activating migration of smooth muscle are often not fully understood. Hedges et al. [186] studied migration of cultured myocytes from the trachea in response to platelet-derived growth factor, interleukin-1 beta and transforming growth factor-beta. They blocked the migration of cells by the use of SB203580 (pyridinyl imidazole) which is an inhibitor of p38 MAPK. They observed during the course of these experiments prolonged phosphorylation of the kinase. Activation of this kinase induced phosphorylation of HSPB1, which could influence the polymerization of F-actin. They used adenovirus-mediated expression of activated mutant MAPK kinase 6b(E), an upstream activator for p38 MAPK, which increased cell migration. However, overexpression of p38alpha MAPK dominant negative mutant and an HSPB1 phosphorylation mutant blocked cell migration completely. The results showed that activation of the p38 MAPK pathway by growth factors and proinflammatory cytokines regulates smooth muscle cell migration and might lead to pathological states involving smooth muscle dysfunction [186,187]. Landry and Hout [117] examined the migration of vascular endothelial cells, and discovered that vascular endothelial migration depends on p38 MAP kinase [188]. In response to activation by serum growth factors and cytokines, p38 MAPK phosphorylates participants of the signaling pathway mediated by HSPB1 [189,170]. Subsequent examination by other groups confirmed that the p38 pathway MAPK/MK2/HSPB1 is necessary for the migration of vascular smooth muscle cells [190], neutrophils [191], fibroblasts [192] and mammary epithelial cells [193]. Other studies have proven that motility of the intracellular pathogen *Listeria monocytogenes* is based on intracellular actin polymerization and may be inhibited by suppression of HSPB1 phosphorylation. The results indicate that activation of the p38 MAPK pathway by growth factors and proinflammatory cytokines regulates smooth muscle cell migration and may be implicated in pathological states involving smooth muscle dysfunction [42,194].

4.4. Functions of HSPB6 in smooth muscle

The increase of myoplasmic Ca^{2+} induces smooth muscle contraction via the activation of myosin light chain kinase (MLCK) and phosphorylation of myosin regulatory light chains (MRLC) [195]. The phosphorylation of MRLC on Ser19 permits actin and myosin interaction, which leads to generation of smooth muscle contraction [196]. HSPB6 plays an opposite role compared to HSPB1 in smooth muscle contraction. The investigation showed that phosphorylated HSPB6 is a significant inhibitor of smooth muscle contraction [196,197]. There are two possible models of this mechanism: cyclic nucleotide dependent (PKA/PKG) depolymerization of F-actin or a direct inhibitory outcome on actomyosin. One of the more important features of HSPB6 in smooth muscle physiology is the phosphorylation of Ser16 by one of the kinases PKA/PKG. The increase in phosphorylation is induced by enlarging the vessel, which has been connected with HSPB6 aggregation in the carotid artery of smooth muscle [179,198]. It is commonly known that cyclic nucleotide-independent relaxation is the main effect of reducing the intracellular level of calcium and dephosphorylation of myosin light chains. However, other studies of smooth muscle relaxation reveal a pathway independent of changes in phosphorylation of myosin light chain [180,199]. The phosphorylation of HSPB1 inhibits HSPB6 phosphorylation and is associated with reduced cyclic nucleotide-dependent vascular smooth muscle relaxation [176].

HSPB6 can also react with 14-3-3 proteins, which bind phosphoproteins. HSPB6 can decrease phosphorylation of cofilin – an actin-binding protein engaged in its disassembly – and disrupt actin in smooth muscle, suggesting that one possible mechanism by which HSPB6 mediates smooth muscle relaxation is by regulation of actin filament dynamics [3,38,200–203]. This is in agreement with the fact that the inhibition of actin polymerization is sufficient to relax smooth muscle, although it is not required [204]. Phosphorylated HSPB6 is a functional smooth muscle relaxant and can be applied to develop new vasodilators and bronchodilators.

5. Pathological aspects of sHSPs

The higher order organization of sHSPs is based on the formation of dynamic oligomers in which subunits undergo exchange. Due to this property sHSPs are able to participate in the retention of cell proteostasis by protecting structure and function of various proteins and thereby prevent different diseases. When sHSPs are perturbed (e.g. by mutation of the cognate genes) their presence may contribute to the disruption of some cellular activities and to the development of disease phenotypes.

Among genes coding for sHSPs, CRYAB undergoes the most frequently genetic alternation that may lead to diverse and

multisystemic diseases. This diversification of phenotypes is a consequence of participation of this multifunctional protein in various cellular processes. Besides HSPB5, other sHSP family members prone to mutations of their genes should be mentioned, i.e. HSPB1, HSPB6 and HSPB7. Mutations affecting HSPB5 influence its intramolecular (e.g. D109H) [205] as well as intermolecular interactions (e.g. R120G) [35,206].

Among disorders in which sHSPs are implicated, desmin-related myopathy (DRM) is one of the best known. It is caused by several mutations in the CRYAB gene, including R120G (also connected with cataract); ΔC13 (464delCT), ΔC15 (450delA), and Q151X mainly affecting both muscular and cardiac tissues [44,207–210].

The arginine residue at position 120 in the ACD is highly conserved and has been shown to be essential for the solubilization and chaperone activity of HSPB5 [44,207]. The functional significance of this residue can be illustrated by the fact that it is present in vertebrates (mouse, zebrafish) as well as in invertebrates (e.g. *D. melanogaster*). Mutation of this residue that leads to DRM that is inherited in an autosomal-dominant manner and causes a loss of the chaperone activity of HSPB5 *in vitro*, and is expected to promote interaction between the mutant protein and the type III IFs. It has been reported that the R120G mutation enhances the binding capacity of HSPB5 for desmin and decreases its dissociation constant [43]. Biochemical and structural studies have revealed that the R120G mutation in HSPB5 disrupts protein secondary, tertiary and quaternary structure. Muscle cell lines transfected with the mutant cDNA encoding HSPB5 showed intracellular aggregates that contain both desmin and HSPB5, which are similar to spheroid inclusion bodies found in DRM patients. In non-pathological conditions a small amount of abnormal desmin interacted with HSPB5, remained soluble, and subsequently degraded. As a consequence of CRYAB mutation, misfolded desmin forms aberrant aggregates and disrupts the integrity of the sarcomere network, thus giving rises to pathological consequences in muscle fibers [211]. It has been suggested that desmin aggregates observed in DRM patients may not be due to direct physical or biochemical interaction between desmin and mutant HSPB5, but rather due to loss of HSPB5 "chaperoning" function [43]. In a knock-in mouse model for R120G mutation of HSPB5 both heterozygous and homozygous mutant mice developed myopathy similar to that in humans carrying the HSPB5 R120G mutation, with insoluble aggregates containing abnormal HSPB5 and desmin [212]. However, how mutated HSPB5 contributes to these pathological states is still not clearly understood. According to some theories, formation of aggregates supervised by, *inter alia*, sHSPs may be a kind of protective mechanism by which organisms eliminate potentially toxic polypeptides whose soluble forms could affect cells in a much more severe manner [213]. Formation of aggregates may also arise through the overloading of a protective mechanism which is no longer able to efficiently eliminate undesirable peptides [214].

Recently, also recessively inherited, a more severe form of HSPB5-related myofibrillar myopathy (MFM) has been identified. The more severe phenotype is probably a result of homozygosity for the mutation (c.343delT). Infant patients affected with disease caused by the relevant mutation show, besides symptoms characteristic of other forms of DRM, also profound muscle stiffness, which may suggest involvement of the CRYAB gene product in modulation of muscle contractility. According to the postulated explanation, abnormal muscle activity may be connected with disruption of the HSPB5 translocation process to the Z-disk during muscle contraction, which may disturb the mechanism responsible for the maintenance and repair of contractile units [215].

Mutation R120G in the CRYAB gene has also been linked with cardiomyopathy. At the molecular level the presence of mutant protein in cardiomyocytes leads to abnormalities in HSPB5 as well as desmin aggregation [209]. It is also associated with reductive stress. In mice overexpression of $HSPB5^{R120G}$ leads to the development of desmin-related cardiomyopathy, which is demonstrated at the molecular level by formation of aggresomes including HSPB5 and desmin, and myofibrillar disorganization [130,209]. Other sHSPs can also be responsible for the development of cardiomyopathic phenotype. For example, HSPB8 is implicated in idiopathic dilated cardiomyopathy [216–218].

An accumulation of misfolded proteins induces proteotoxic stress and invokes in the cell a stress response connected with elevation in the expression levels of different HSPs, including HspB1, HspB5, HspA1–Hsp70 and Hsp90 alpha [130,219]. Attenuation of HSP expression contributes to restoration of redox balance; however, the presence of mutant protein shifts the equilibrium toward reductive stress.

The presence of aggregates formed due to the presence of mutated HSPB5 is accompanied by high oxidative stress. This occurs via the Nrf2 pathway [220]. The presence of toxic complexes has additional effects such as sequestration of different proteins crucial for protein homeostasis maintenance, including HSPB1, KEAP1 (a negative regulator of Nrf2) and G6PD. It also leads to the disruption of cardiac sarcomeric units [221].

Furthermore, mutations in binding partners of sHSPs may also affect functions of this chaperone leading to the development of pathological phenotypes similar to those deriving from abnormalities in genes coding for sHSPs. For example, mutation (P209L) of BAG3 in the region responsible for binding HSPB8 and HSPB6 results in childhood muscular dystrophy with progressive limb and axial muscle weakness. Affected patients develop cardiomyopathy and peripheral neuropathy symptoms [222,223].

Altered expression of some sHSPs was observed in different cardiac pathologies, e.g. HSPB1 level is changed in patients with congestive heart failure [224]. Acute ischemia also causes upregulation of HSPB1 expression in myocardial tissue [225], which is connected with its role in cardiac muscle protection from different stressors [136]. Distribution of HSPB1 is also changed in some pathological states such as cardioplegia, in which HSPB1 is translocated to the myofilaments [226].

In the case of muscle activity, the protein quality control system supervises the fate of sarcomeric components on several levels, which include: correct folding of newly synthesized proteins, their incorporation into existing filaments, disks or other structures, exchange of individual elements without affecting the functioning of the others, and with the maintenance of entire muscle integrity. As members of the PQC system sHSPs participate in all these processes in a direct or indirect manner. However, their main goal seems to be protection of muscle components from unfavorable changes that are consequences of activity of different stress agents. Thereby the necessity of understanding functioning of sHSPs both under normal and pathological conditions seems to be reasonable. Furthermore, a better understanding of this issue should allow scientists to elaborate effective medical tools for treatment of pathological states connected with disrupted chaperones.

6. Perspectives

The multifunctional character of sHSPs leads to their involvement in a variety of cellular processes. In normal conditions they act as housekeeping proteins, whereas in stress conditions they help maintain or restore cell homeostasis. In pathological states, such as cancer and ischemia, their multifunctional nature seems to present a serious obstacle hindering their potential usage in medical treatment of different pathological states. A successful and tissue-specific treatment demands accurate drug administration in a way that allows minimizing side effects (e.g. in cancer therapy an effect on cardiac function due to the increased level

of oxidative stress). This in turn creates an urgent need for further investigation of the properties of sHSPs, their binding partners (*i.a.* ATP-dependent chaperones and their co-chaperones), analysis of the signaling pathways modulated by them, and interactions of genes encoding them with different transcription factors that act in a stage- and tissue-dependent manner. Additionally, since the chaperone activity of sHSPs is regulated by post-translational modifications such as phosphorylation (whose impairment contributes to various pathological conditions including cancer), these modulating functions require a detailed elucidation. Moreover, one of unanswered question regarding these proteins is: which functions of different sHSPs overlap and which are distinct in the developing and adult tissues (including all three types of muscle) in normal as well as pathological conditions? Solution of these issues would allow us to decipher mechanisms of action of sHSPs in various tissues and in the whole organism. Besides expansion of the basic knowledge about sHSPs, this might also allow devising a tissue-specific delivery technique for the existing sHSP-based therapeutic agents as well as the development of new competitive sHSP inhibitors which meet the challenges of contemporary medicine.

As mentioned above, one of the current directions in sHSP research is focused on examination of their usage as target proteins in medical approaches. For example, in the case of muscle regenerative medicine, application of HSPB6 has given promising results [227]. Injection of mesenchymal cells overexpressing this protein in ischemic hearts improved survival of cardiomyocytes. This is due to the ability of overexpressed HSPB6 to activate the Akt pathway-dependent secretion of growth factors such as VEGF, FGF2 and IGF1 [227]. Controlled overexpression of HSPB1 via pharmacological inducers such as Herbimycin A and Geranylgeranylacetone (GGA) in tissues prone to ischemic damage also seems to be a reasonable therapeutic approach to enhancing its cardioprotective activity [228].

Also the pro-oncogenic role of some sHSPs, e.g. HSPB1 or HSPB8, complements (but not exhausts) the long list of their activities and is the subject of cancer research. Overexpression of these proteins was confirmed in different types of tumors (see chapter 1.2). Their connections with tumorigenesis consists in involvement in the development of drug resistance – a disadvantageous phenomenon which is one of the most important reasons for low effectiveness of chemotherapy. Elimination of sHSPs, in view of their cytoprotective properties (which leads to cancer cell chemosensitization and radiosensitization), seems to be a reasonable strategy in the battle against cancer. To achieve this purpose, a broad spectrum of potential therapeutic agents is used. They include three distinct classes of molecules: small molecule inhibitors, antisense oligonucleotides, and protein aptamers. It is noteworthy that these novel sHSP-based drugs have already found (or will find in the nearest future) application in therapies for other types of disease.

Finally, in chapter 1 we have emphasized the evolutionary conservation of sHSPs, which are synthesized in almost all organisms. This makes it possible to compare the results of sHSP studies obtained from different models (invertebrate as well as vertebrate). Also the pathological implications of different sHSPs seem to be evolutionarily conserved (e.g. the amino acid residue R120 of HSPB5 altered in human DRM disease is conserved in other vertebrates including mouse and zebrafish, as well as in the invertebrate *D. melanogaster*. Therefore, creation of human disease models using mouse, fly, zebrafish, and other animals is justifiable and improves our understanding of the etiology of pathological phenotypes.

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