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Heat stress induces mitochondrial adaptations in skeletal muscle

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Abstract Heat stress treatment is a classic physical therapy, which is employed in the orthopedic field. In the field of physical fitness/sports science, morphological changes of skeletal muscle by heat stress have been well studied. In recent years, energy metabolic adaptations by heat stress have also been actively studied. In this review, we provide an overview of recent findings on heat stress-induced mitochondrial adaptations in skeletal muscles, and further discuss our unpublished data and recent findings in related research fields. First, we summarized heat stress-induced positive regulation of mitochondrial content and its underlying molecular mechanisms from perspectives of mitochondrial biogenesis and degradation. Consequently, we reviewed beneficial effects of heat stress on mitochondrial health in disused and aged muscles, focusing on mitochondrial stress response at the organelle level (mitochondrial selective autophagy; mitophagy) and molecular level (mitochondrial unfolded protein response). Finally, we overviewed future directions to better understand heat stress-induced mitochondrial adaptations in skeletal muscle.

Keywords : mitochondria, skeletal muscle, autophagy, unfolded protein response, heat stress

Brief history of research on heat stress in skeletal muscle

Heat stress treatment is a physical therapy that has been used since ancient times. Interestingly, literature recommending health promotion using heat stress was published at least 200 years ago in Japan. Currently, heat stress is generally used in the medical field, especially in orthopedics. However, the mechanisms underlying the health promotion effects of heat stress have not been sufficiently understood. In the field of physical fitness/sports science, morphological changes in skeletal muscle by heat stress have been well studied. For example, it has been reported that heat stress promotes the regeneration of injured rat skeletal muscle (hot pack, 42ºC, 20 min[1] / heat exposure, 41ºC, 60 min[2]), and suppresses hindlimb unloading-induced skeletal muscle atrophy (heat exposure, 42ºC, 60 min[3]). Increases in heat shock proteins (HSPs), molecular chaperones, by heat stress and the concomitant enhancement of proteostasis capacity can be, at least in part, involved in potential mechanisms. Heat stress protocols should be carefully selected according to the purpose of each research project undertaken. In fact, mode, temperature, and time of heat stress were varied between studies. As will be described later, even if there is a slight difference in heat stress protocol, there is a possibility of causing the opposite cellular response (see later paragraph discussing "*AMPK response by heat stress*"). Therefore, to understand adaptations of skeletal muscle by heat stress, it is necessary to carefully consider protocols. Such findings and methodology of heat stress were reviewed by Naito and colleagues[4].

In recent years, energy metabolic adaptations by heat stress have also been actively studied. Therapeutic effects of heat stress on insulin resistance have been reported in type 2 diabetes of rodents (hindlimb hot bathing, 41ºC, 20 min)[5] and patients (Hot tub bathing, 37.8-41.0ºC, 30 min/day, 6 days/week, 3 weeks)[6], mediated by up-regulated glucose uptake in skeletal muscles, based on a mechanistic study investigating isolated skeletal muscle (42ºC, 30 min)[7]. In addition to glucose metabolism, several lines of evidence demonstrated various adaptabilities of mitochondria-centered oxidative metabolism by heat stress. In this review article, we briefly summarize research outcomes on adaptations of skeletal muscle mitochondria by heat stress, and deepen the discussion with our unpublished data and studies from related research fields.

Heat stress induces mitochondrial biogenesis in skeletal muscles

Mitochondria are intracellular organelles contributing to various biological processes such as energy production, thermogenesis, cell death, ion handling and secretion of peptides. Holloszy first discovered that exercise train-

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ing increases mitochondrial content in skeletal muscle[8]. To date, it is well accepted that exercise training is the most effective way to increase mitochondrial content and function. Increased mitochondrial content and its related increased oxidative capacity contribute to exercise performance at sub-maximal intensity via glycogen sparing[9]. Furthermore, recent emerging evidence shows that mitochondrial loss and dysfunction can mediate disuse-induced skeletal muscle atrophy[10-12]. The biological basis and significance of mitochondrial homeostasis in exercised, disuse atrophied, and aged muscles are reviewed in several papers by Hood and colleagues[13-17].

The pioneering study by Liu and Brooks demonstrated that heat stress induces mitochondrial biogenesis in C2C12 myotubes (40ºC, 60 min/day, 5 days)[18]. Moreover, Yamaguchi and coworkers have reported that heat stress promotes differentiation into slow/oxidative muscle fibers in human myoblasts (39ºC, continuous, 7 days)[19]. These *in vitro* studies strongly suggest that heat stress can modulate oxidative metabolism. To translate these findings in *in vitro* to *in vivo* skeletal muscle, we first conducted research on whether or not heat stress increases mitochondrial content in mouse skeletal muscles. As expected, we found that heat stress does increase mitochondrial content, based on increased the protein content of mitochondrial OXPHOS enzymes and enzyme activity of citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (3-HAD) (exposing mice to a hot environment, 40ºC, 30 min, 5 days/week, 3 weeks)[20]. Importantly, these mitochondrial adaptations were observed in both plantaris (fast fiber dominant) and soleus muscle (slow fiber dominant). Therefore, heat stress-induced mitochondrial biogenesis could be independent of muscle type. Furthermore, as an advantage tested *in vivo*, we also demonstrated that post-exercise heat stress additively enhances endurance training-induced mitochondrial adaptations. Incidentally, a previous study reported that a high-fat diet feeding also leads to mitochondrial biogenesis in skeletal muscle[21]. However, in another high-fat diet study, heat stress canceled mitochondrial biogenesis in rat skeletal muscle (hindlimbs hot bathing, 41ºC, 20 min)[5], indicating that the additive effects of heat stress on mitochondrial adaptations are not universally observed. Thus, it could be notable that there are additive effects of heat stress on exercise training-induced mitochondrial biogenesis. Other groups reported that exposure to a hot environment after running enhances endurance exercise capacity in humans (humid sauna, 89.9ºC, 31 min, 3 weeks)[22], supporting our observation on mitochondrial adaptations in mice. Taken together, we conclude that heat stress induces mitochondrial biogenesis *in vivo* skeletal muscles similar to *in vitro* studies. A series of evidence suggest that there is further applicability of heat stress-induced mitochondrial adaptations in *in vivo* skeletal muscles.

Heat stress activates regulatory factors of mitochondrial biogenesis

Mitochondrial biogenesis is regulated by several steps including transcription, translation, and post-translational processing[23]. Among them, the transcriptional regulating stage of mitochondrial biogenesis has been most studied. Since mitochondria-associated genes are encoded by both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA), functionally coordinated gene transcriptions are important. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is known as a master regulator of mitochondrial biogenesis and oxidative metabolism[24]. Importantly, PGC-1α can contribute to mitochondrial gene transcription in two different ways: 1) via activation of PGC-1α with no change in PGC-1α content[25], and 2) via increased PGC-1α content[25,26].

Activated PGC-1α translocates to the nucleus and mitochondria from the cytosol, and then promotes both transcription of nDNA and mtDNA-encoded mitochondria-associated genes by co-binding with transcriptional factors (TF; *e.g.* Peroxisome proliferator-activated receptor gamma [PPARγ], Nuclear respiratory factor 1/2 [NRF1/2], p53, and/or mitochondrial transcriptional factor A [TFAM]). We have recently found that a single bout of heat stress caused PGC-1α to translocate to both the nucleus and mitochondria (concomitantly with translocation of TFAM) at 3 hours after treatment (unpublished data). Furthermore, we also found that transcripts of mitochondrial enzymes encoded by both nDNA (also termed as nuclear genes encoding mitochondrial proteins [NuGEMPs]) and mtDNA are increased by heat stress (unpublished data). These findings suggest that heat stress promotes transcription of mitochondria-related genes with activation of PGC-1α.

In response to cellular stress, the transcription factor HSF1 is activated, forms a trimer and translocates to the nucleus from the cytosol[4]. Activated HSF1 binds to a specific sequence in promoter regions of DNA, and promotes transcription of the target genes. This particular sequence is described as heat shock element (HSE) and is present in the promoter region of many HSPs. The activation of HSF1 and consequent induction of HSP are described as heat shock response (HSR). The recent computational study showed that HSE was found in the promoter region of PGC-1α[27], suggesting transcription of the PGC-1α gene can also be regulated by HSF1. Actually, another recent report has shown that HSF1 positively regulates PGC-1α transcription in adipose tissues and skeletal muscles[28]. Interestingly, although HSR is thought to be the basic preservative and essential process of cell protection, HSF1-dependent PGC-1α transcription depends on tissue type (*e.g.* not observed in brain, kidney and liver). Returning to our observations, we also found that not only PGC-1α activation, but also heat stress leads to HSF1 nuclear translocation and increases PGC-1α transcription

(unpublished data). This evidence strongly suggests that the mechanism underlying heat stress-induced mitochondrial biogenesis can be explained by both activation and induction of PGC-1α. On the other hand, a recent study has shown that PGC-1α co-binds with HSF1 and promotes transcription of HSP[29]. These findings, of the activation of PGC-1α -centered transcriptional networks in response to heat stress, cellular protection and oxidative energy metabolism, could be more closely related than our conventional understanding.

Activation and/or induction of PGC-1α is induced by activation of various upstream signaling molecules. For example, it is clarified that translocation/induction of PGC-1α is mediated by activation of protein kinase such as AMP-activated protein kinase (AMPK)[30], p38 mitogen-activated protein kinase (p38 MAPK)[31], calcium/calmodulin-dependent protein kinase II (CaMK II)[32] and mammalian/mechanistic target of rapamycin complex 1 (mTORC1)[33]. Therefore, in order to elucidate the mechanism inducing activation of PGC-1α by heat stress, we examined the effect of heat stress on the activation status of upstream regulatory factors of PGC-1α. As a result, it was revealed that p38 MAPK and mTORC1 were activated by heat stress[20]. Interestingly, it was shown that heat stress suppresses the activity of AMPK. Some studies *in vitro* demonstrated that responses of these kinases are required for HSF1 activation or HSP induction[34-36]. Importantly, there is no unified view on the effect of heat stress on AMPK. Previous studies on isolated muscles have been reported to activate AMPK by heat stress (42ºC, 10 min)[37]. In addition, opinions are divided on research targeting cultured cells (Activation[18]: 40ºC, 30 min; Inactivation[34]: 42ºC, 30 min). It is notable that AMPK can be both activated or inactivated by heat stress not only in muscle tissues, but also in cultured cells. According to these reports, an opposite response of AMPK by heat stress can be explained by differences in heat stress conditions such as temperature and time, rather than by differences between *in vitro* and *in vivo* such as involvement of inter-tissue interaction via humoral factors. Since the activity of AMPK not only controls mitochondrial biogenesis but also glucose metabolism, cell cycle and proteostasis[38], understanding the bipolar change of AMPK activity by heat is considered an important future direction for research.

Compared to our understanding of transcriptional regulation, translational and post-translational regulation of mitochondrial biogenesis are less understood in the research field of exercise biology[23]. Nuclear-encoded mitochondrial proteins, including mitochondrial transcription factors, are translated and synthesized in the cytosol as precursor proteins. It has been clarified that newly synthesized mitochondrial precursor proteins are transported into the mitochondria and refolded by mtHSP and HSP72[39]. Heat stress dramatically increases mtHSP and HSP72 protein contents in skeletal muscle[20,40]. One could speculate that an increase in mtHSP and HSP72 may promote transportation efficiency into the mitochondria and the folding process and then enhance mitochondrial adaptations. Actually, genetic overexpression of HSP72 increases mitochondrial content in skeletal muscle[41]. Importantly, in that study, no change in PGC-1α expression was observed in HSP72 overexpressed muscles[41].

In summary, the mechanisms underlying heat stress-induced mitochondrial biogenesis can be explained by multiple regulatory steps. A schematic summary is shown in Fig. 1. Importantly, they are likely to differ from the mechanisms underlying exercise training-induced mitochondrial biogenesis.

Heat stress attenuates mitochondrial loss due to experimental muscle disuse

It has been reported that skeletal muscle atrophy is suppressed by heat stress as described above. However, its molecular mechanism has not been fully elucidated. Recently it has been reported that mitochondrial dysfunction and/or loss can cause skeletal muscle atrophy[10-12]. Based on our previous findings, we investigated whether heat stress suppresses the decrease in mitochondria, concomitantly with skeletal muscle atrophy, in order to elucidate the mechanism by which heat stress suppresses skeletal muscle atrophy. Ten days of sciatic nerve resection (denervation; an experimental muscle disuse model) reduced skeletal muscle weight and mitochondrial content, but their reduction was suppressed by seven days of heat stress treatment during the denervation period[42]. Since denervation-induced mitochondrial loss can be explained by a decrease in PGC-1α, we examined the possibility that heat stress attenuates the reduction of PGC-1α by denervation. As a result, heat stress did not suppress the decrease of PGC-1α due to denervation[42]. In addition, although glucose transporter 4 (GLUT4) and fatty acid transporter/cluster of differentiation 36 (FAT/CD36), targets of PGC-1α other than mitochondria, also showed a decrease due to denervation, an improvement effect by heat stress was not observed[42]. These results suggest that the maintenance of the mitochondrial content by heat stress may be related to the mitochondrial-specific pathway rather than PGC-1α dependent pathway.

Heat stress attenuates denervation-activated mitophagic flux

The content of mitochondria is determined by the net balance between mitochondrial biogenesis and degradation. Dysfunctional mitochondria (*e.g.* ROS accumulation, depolarized membrane potential [ΔΨm]) produce ROS, and ultimately impair cellular health. For maintenance of cellular homeostasis, dysfunctional mitochondria are degraded by selective autophagy. Autophagy is a major proteolytic system; proteins to be degraded are

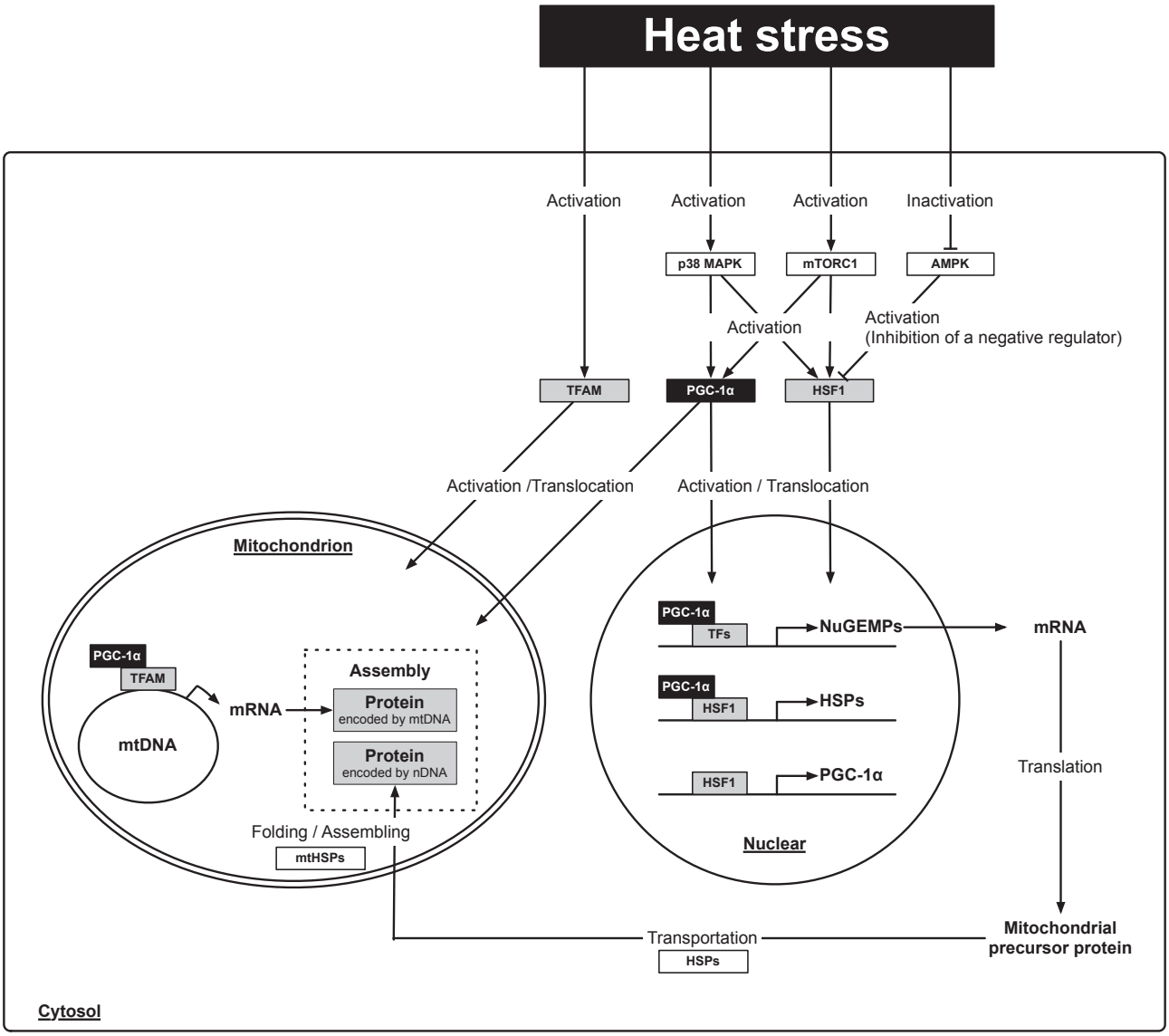

Fig. 1 Heat stress promotes mitochondrial biogenesis in skeletal muscle. Heat stress activates intra-cellular signal transduction which subsequently promotes transcription of mitochondrial-related genes encoded by both nDNA and mtDNA. Furthermore, increased cytosolic and mitochondrial molecular chaperones can also contribute to mitochondrial biogenesis by enhancing capacities of post-translational protein handling. (also see section "*Heat stress activates regulatory factors of mitochondrial biogenesis*" in text).

sequestered into large double-membrane vesicles called autophagosomes. Dr. Yoshinori Ohsumi, a Japanese biologist, won the Nobel Prize in Physiology or Medicine in 2016 for his discovery of autophagy and its regulating mechanism[43,44]. At first, autophagy was discovered as a non-selective bulk proteolytic system. However, to date, some types of selective autophagy have been found such as mitochondria (mitophagy), and nuclear (nucleophagy).

Denervation increases mitophagic flux[45]. In contrast, we found that heat stress attenuates denervation-activated mitophagic flux[42]. Mitophagy is roughly divided into two processes of 1) ubiquitination of mitochondrial proteins (*i.e*. labeling the mitochondria to be degraded) and 2) autophagosome formation. We demonstrated that the denervation-increased proteins involved in mitochondrial ubiquitination (mitochondrial E3 Ub ligase Parkin), actual content of Ub-conjugated mitochondria and adaptor protein (mitochondrial p62) between Ub-conjugated mitochondrial proteins and autophagosome, are reduced by heat stress[42]. Regulation of autophagosome formation is classified into three steps: induction, nucleation and elongation. Heat stress did not affect the major proteins involved in the induction of autophagy (active forms of AMPK, mTOR, UNC-51-like kinase 1 [ULK1], and autophagy-related gene [ATG] 13) and the nucleation steps (ATG14 and Beclin 1). In contrast, heat stress attenuated an increase, due to denervation, in the protein involved in the elongation step (ATG5-12 conjugate and ATG16L)[42]. How are only the proteins related to the elongation step of autophagosome formation suppressed by heat stress? Unlike other ATGs, ATG5-12 conjugate and ATG16L were suggested to be regulated by the activating transcription

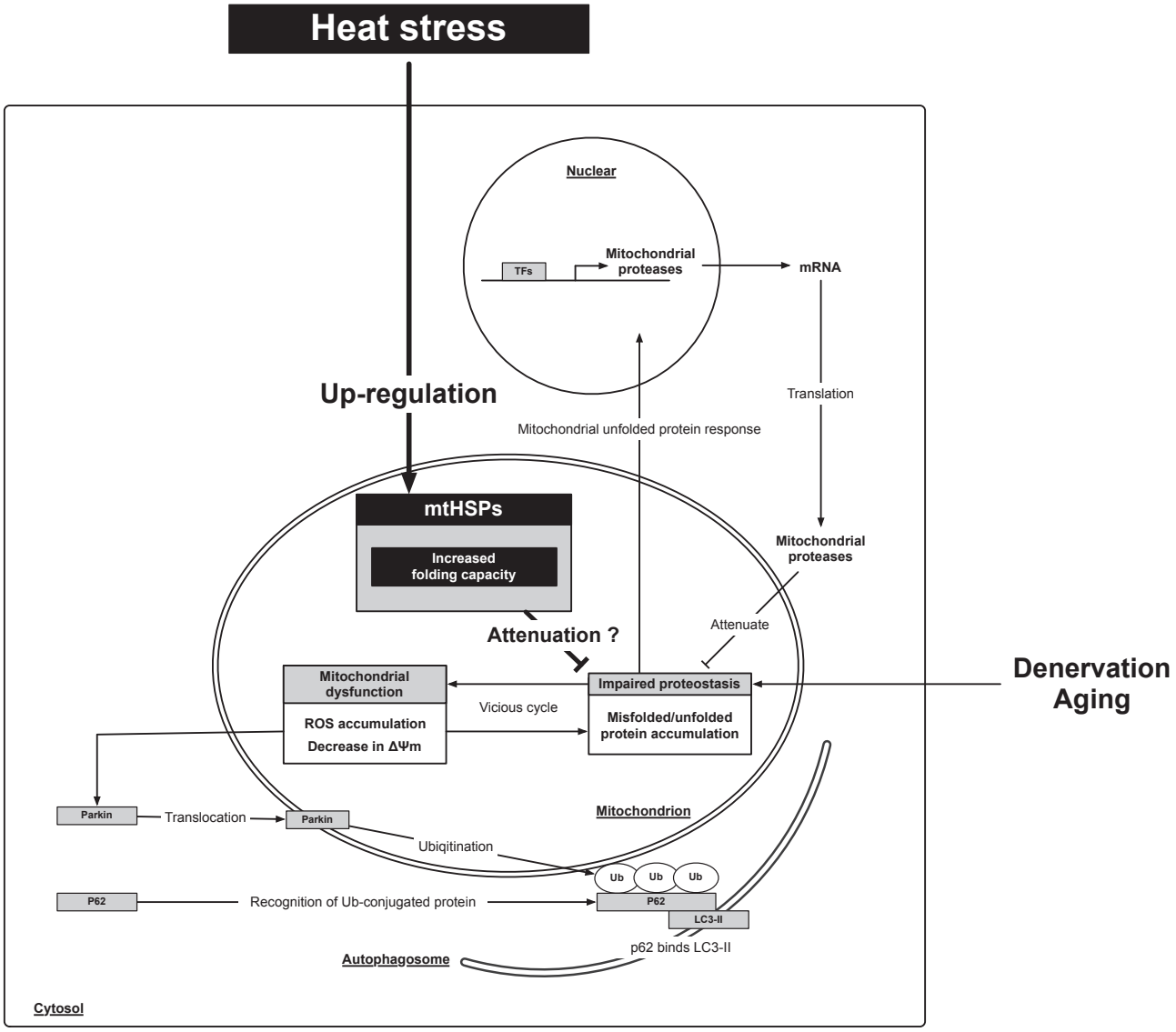

Fig. 2 Possible mechanisms underlying heat stress-induced improvement of mitochondrial stress and stress responses in denervated and aged muscle. Denervation and aging increase misfolded/unfolded proteins in mitochondria, which subsequently promotes mitochondrial dysfunction such as ROS accumulations and decrease in mitochondrial membrane potential. To combat mitochondrial dysfunction and its related toxicity, mitochondrial quality control machineries are activated at organelle (mitophagy) and molecular level (mitochondrial unfolded protein response). Heat stress attenuates oxidative stress and mitochondrial stress responses in denervated and aged muscles. Heat stress-induced mtHSPs and increased chaperoning capacity can be considered as its underlying possible mechanisms. (also see section "*Heat stress improves mitochondrial health*" in text).

factor 4 (ATF4) from a previous study[46] and databases. It is known that ATF4 is activated by increased endoplasmic reticulum (ER) stress. We expectedly found that marker proteins of ER stress (CCAAT-enhancer-binding protein homologous protein [CHOP], cleaved Caspase12, glucose-regulated protein [GRP] 78, protein disulfide isomerase [PDI] and Calnexin) are increased by denervation (unpublished data). However, no improving effects by heat stress on these ER stress markers were observed (unpublished data). Therefore, it seems that a pathway other than ER stress contributed to the pathway that suppressed the increase of protein involved in the elongation process of autophagosome. However, at the present time, specific targets have not been identified. In summary, the molecular basis of suppression of denervation-activated mitophagy by heat stress is explained by 1) a reduction of the ubiquitination of mitochondrial proteins and 2) a decrease in proteins involved in the elongation process of autophagosome formation.

Heat stress improves mitochondrial health

It should be carefully interpreted that heat stress suppresses denervation-activated mitochondrial breakdown. There is room for the following two interpretations: 1) Heat stress directly attenuates the degradation of mitochondria, which raises a serious concern that mitochondria retained by heat stress are unhealthy; 2) Degrada-

tion of mitochondria is consequently suppressed, which means that heat stress primarily improves mitochondrial health and then it is no longer necessary to breakdown mitochondria. In order to clarify these two possibilities, we examined mitochondrial stress and stress response another than mitophagy.

In the condition that mitochondrial stress (*e.g.* accumulation of oxidative stress and/or unfolded/damaged proteins) is progressing, retrograde signaling from mitochondria to nuclear is activated to increase mitochondrial proteases and chaperones, which is known as mitochondrial unfolded protein response (UPR^{mt})[47]. In this way, mitochondrial quality is controlled at not only the organelle level (mitophagy), but also molecular level (UPR^{mt}). We found that mitochondrial oxidative stress (mitochondrial 4-hydroxynonenal [4-HNE] proteins) and mitochondrial proteases (ATP-dependent Clp protease proteolytic subunit [ClpP] and high-temperature requirement serin peptidase 2 [HtrA2]) were increased by denervation (unpublished). Consistent with the adaptations of mitophagy, heat stress counteracted denervation-induced up-regulation of mitochondrial oxidative stress and proteases. Incidentally, situations in which mitochondrial stress occur include not only muscle disuse, but aging. We also demonstrated that mitochondrial stress responses, inductions of mitochondrial E3 ubiquitin ligase (Parkin) and mitochondrial proteases (CLPP and HtrA2), were increased in aged muscles, concomitantly with increased oxidative stress. In simulated denervated conditions, daily heat stress intervention recovered these biomarker proteins to young mice levels[48]. Taken together, heat stress can improve mitochondrial health under conditions of mitochondrial stress.

Why does heat stress maintain mitochondrial health? We considered that increased mitochondrial localized chaperones (*i.e.* mtHSPs) and subsequent enhanced proteostasis capacity are mechanism candidates. In recent years, increased mitochondrial stress could be promoted by disruption of mitochondrial proteostasis. Several lines of evidence from non-muscle cultured cells demonstrated that genetic overexpression of HSP60 attenuates oxidative stress accumulation[49]. In a series of our studies, we observed that not only cytoplasmic HSP (*e.g.* HSP72), but also mitochondrial HSP (*e.g.* HSP60, mtHSP70, and TNF receptor-associated protein 1 [TRAP-1]) were dramatically increased by heat stress treatment[20,43,48]. Furthermore, an interesting observation was recently reported that HSP72, conventionally considered as cytosolic HSP, translocates to mitochnodria from cytosol and interacts with Parkin in response to increased mitochondrial stress[50]. Therefore, in addition to an assay of protein, analyzing properties of HSPs at the post-translational level (*e.g.* sub-cellular localization, physical and functional interactions between other proteins, and functional capacity of protein folding) could help to promote our understanding. A schematic summary of heat stress-induced improvement of mitochondrial health is shown in Fig. 2.

Future direction

Mitochondria physically and functionally interact with other organelles and molecules in the cell. Therefore, our careful attention should be paid to potential adaptations of other molecules and organelles as well as mitochondria by heat stress. This approach could help us to better understand the heat stress-induced mitochondrial adaptations. Our ongoing study aimed to elucidate new cellular adaptations by heat stress and identify new candidates by using transcriptome and bioinformatic techniques. We are working on elucidating the molecular basis and physiological significance of this novel cellular response, and the linkage with mitochondria.

Conflicts of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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