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Possible Involvement of Mitochondrial Reactive Oxygen Species Production in Protein Degradation Induced by Heat Stress in Avian Muscle Cells

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Heat stress (HS) stimulates mitochondrial reactive oxygen species (ROS) production and protein degradation in skeletal muscle. The present study investigated the stimulatory effects of HS-induced mitochondrial ROS production on the ubiquitin-proteasome protein degradation system in primary cultured avian muscle cells. Cells were isolated from the breast muscle of neonatal chicks, and then grown for 48 h. Thereafter, the cells were subjected to 37℃ or 41℃ (HS). Exposure to 6 h of HS treatment significantly decreased the cellular protein content compared to that of normal cells, an effect was completely suppressed by the addition of a proteasome-specific inhibitor. Whereas the mRNA levels of the 20S proteasome C2 subunit, which is one of the subunits of the 26S proteasome, did not change at any time during HS treatment (1, 3, 6 h), the mRNA levels of atrogin-1 and muscle ring-finger protein 1, both of which are muscle-specific ubiquitin ligases, increased after 1 h of HS but then decreased to near-normal values with time. Intracellular ROS production (the sum of H_2O_2, hydroxyl radicals, peroxyl radicals, peroxynitrite) did not change in the 1 h HS-exposed cells, but was significantly increased after 3 h and 6 h of HS. Mitochondrial superoxide production was significantly increased after 1 h of HS, which might increase the mRNA expression of ubiquitin ligase in muscle cells. In cells pretreated with 4-hydroxy TEMPO, which is able to decrease mitochondrial superoxide production, the increases in mitochondrial superoxide production and ubiquitin ligase mRNA levels observed after 1 h of HS were suppressed. The protein content of these cells was not decreased, which was observed after the longest period of HS (6 h). These findings suggest that mitochondrial superoxide production may play an important role in activating the ubiquitin-proteasome system, probably via the induction of ubiquitin ligases, in HS-exposed muscle cells.

Key words: atrogin-1, 4-Hydroxy Tempo, mitochondria superoxide, MuRF1, skeletal muscle cells, ubiquitin-proteasome system

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Introduction

As birds lack sweat glands and are covered with feathers, heat dissipation from their body surface is restricted. Therefore, birds face the threat of hyperthermia. Several physiological and pathological stimuli such as exposure to high temperature (referred to as heat stress, HS), excessive inflammation and sepsis are known to cause hyperthermia in animals. In birds reared under HS conditions, plasma corticosterone levels are increased (Yunianto *et al*., 1997; Willemsen *et al*., 2011), which may contribute to the rapid turnover of muscle protein (Yunianto *et al*., 1997). Several studies have also reported that HS treatment of cultured muscle cells results in an enhancement of protein degradation (Baracos *et al*., 1984; Luo *et al*., 2000; Nakashima *et al*., 2004a), which allows us to postulate that an intracellular intermediator could play a role in driving protein degradation in HS-exposed cells.

Overproduction of reactive oxygen species (ROS) is one of the hallmarks of HS (Zuo *et al*., 2000; Wang *et al*., 2013), and our previous studies have shown that this overproduction mainly occurs in the mitochondria (Mujahid *et al*., 2005; Kikusato and Toyomizu, 2013a). Recent research has proposed that mitochondrial ROS play an important role in intracellular signal transduction (Finkel, 2012), through which they also seem to contribute to protein catabolism (Li *et al*., 2003a; Gilliam *et al*., 2012; Rahman *et al*., 2014). The ubiquitin-proteasome system is a major proteolytic pathway within cells, and comprises two different processes, protein ubiquitination and proteasomal degradation. In these

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processes, proteins undergo ubiquitination via a series of reactions involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3), before being subsequently decomposed by the 26S proteasome. It has been reported that HS activates this system, thereby contributing to protein degradation in skeletal muscle cells (Morita *et al*., 1996; Luo *et al*., 2000; Nakashima *et al*., 2004). Based on these lines of evidence, it can be hypothesized that mitochondrial ROS production may be a factor that activates the ubiquitin-proteasome system under HS conditions. In support of this idea, our previous study found that antioxidant treatment reverses the decrease in cellular protein content that occurs due to HS treatment (Yoshida *et al*., 2013).

To assess the stimulatory effect of mitochondrial ROS on protein degradation in HS-exposed cells, we examined concomitant changes in cellular protein content, the mRNA levels of ubiquitin-proteasome-related genes and mitochondrial ROS production levels in response to different periods of HS treatment, using primary cultured avian skeletal muscle cells. We also measured the changes in cellular protein levels in response to an antioxidant which is able to reduce mitochondrial superoxide, the major radical species generated in this organelle. Here, we demonstrate that mitochondrial superoxide production may play an important role in activating the ubiquitin-proteasome protein degradation system, thereby decreasing the protein content of HS-exposed muscle cells.

Materials and Methods

Ethics Statement

The Animal Care and Use Committee of the Graduate School of Agricultural Science, Tohoku University, approved all procedures, and every effort was made to minimize pain or discomfort to the animals.

Cell Culture

Skeletal muscle cells were isolated from the *superficial pectoralis* muscles of male neonatal chicks. Five 0-day-old male chicks (Ross strain, *Gallus gallus domesticus*) were obtained from a commercial hatchery (Matsumoto Poultry Farms and Hatcheries Co., Ltd., Zao, Miyagi, Japan). The muscles were dissected and digested with a mixture of collagenase (1 mg/ml) and dispase (1000 U/m*l*) for 20 min at 37℃. Cells were collected by centrifugation, washed and resuspended in basal medium (80% Dulbecco's Modified Eagle's Medium (DMEM), 20% M199) supplemented with 10% fetal bovine serum (FBS), 1.5×10^5 U/*l* penicillin and 0.15 g/*l* streptomycin. The cell suspension was transferred to ϕ90 mm-dishes to allow fibroblast attachment, and unattached cells were then collected. The cells were seeded onto collagen Type I-coated ϕ60 mm-dishes or 24-well microplates at a density of 4.5×10^4 cells/cm^2 for each measurement, and were incubated for 48 h at 37℃ under 95% air/5% CO_2 until the cells reached sub-confluence. For the subsequent experiments, the cells were incubated in the medium without FBS and exposed to normal (37℃) or HS (41℃) conditions for a given time (1, 3 or 6 h). Although rectal temperature of chicken is around 41℃, the present study adopted general incubation temperature (37℃) as usually used for elucidating avian muscle metabolisms (Nakashima *et al*., 2004a, 2004b; Joubert *et al*., 2011; Sato *et al*., 2012).

Measurement of Intracellular ROS Production and Mitochondrial Superoxide Production

Intracellular ROS production in muscle cells was fluorometrically determined using 5-(and-6) -chloromethyl-2'7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2 DCFDA, Life Technologies, San Diego, CA, USA) as previously described (Yoshida *et al*., 2013). CM-H_2DCFDA undergoes deacetylation by cytosolic esterases to form dichlorodihydrofluorescein. This then reacts with ROS, such as hydrogen peroxide, hydroxyl radicals, peroxyl radicals and peroxynitrite, to give rise to fluorescein. Mitochondrial superoxide production was detected using MitoSOX^{TM} Red Mitochondrial Superoxide Indicator (Life Technologies, San Diego, CA, USA), which is a fluorescent dye for the highly selective detection of mitochondrial superoxide within cells. After HS treatment, the medium was replaced with 37℃- or 41℃ -prewarmed Hanks' balanced salt solution (HBSS) supplemented with either 5 μM CM-H_2 DCFDA or 5 μM MitoSOX^{TM} Red, and each generation rate was measured as the change in fluorescence at excitation and emission wavelengths (λex/λem) of 485 nm/538 nm for CM-H_2DCFDA and 510 nm/590 nm for MitoSOX^{TM} Red, for 30 min each. The assay was carried out on a computer-controlled fluorescence microplate reader (Ascent Fluoroskan, Thermo Scientific, Waltham, MA, USA) at 37℃ or 41℃. Both dyes were dissolved in dimethyl sulfoxide (DMSO), and this was then added to HBSS to make a 0.1% solution. The fluorescence intensity was normalized against the protein value.

An antioxidant, 4-hydoxy TEMPO (Tmp) (5 mM), which is able to permeate the mitochondrial membrane, mimic superoxide dismutase (Krishna *et al*., 1996) and act as a superoxide scavenger (Laight *et al*., 1997), was added to the incubation medium with FBS for 1 h prior to HS treatment to examine the involvement of superoxide in the ubiquitin-proteasome system under HS conditions.

Measurement of Cellular Protein Content

The cells were washed twice with phosphate buffered saline (PBS), collected with 2% sodium dodecyl sulfate, and stored at −20℃ until required. The protein content was determined using the micro bicinchoninic acid (microBCA) assay (Thermo Scientific, Waltham, MA, USA), with bovine serum albumin as the standard. The absorbance at 550 nm wave length was detected using an iMark^{TM} Microplate Reader (Bio-Rad, Hercules, CA, USA).

Proteasome Inhibitor I (20 μM) (Merck Millipore, Billerica, MA, USA) was used to inhibit the chymotrypsin-like activity of the 20S proteasome (Figueiredo-Pereira *et al*., 1994), which is a multicatalytic proteinase unit of the 26S proteasome. The inhibitor was added to the incubation medium without FBS to examine its effect on cellular protein content. The inhibitor was dissolved in DMSO, and this was then added to the above medium to make a 0.1% solution prior to HS treatment.

Quantification of mRNA Expression using Real-time Polymerase Chain Reaction (RT-PCR)

The effects of HS treatment on the mRNA levels of genes related to the ubiquitin-proteasome system, such as atrogin-1, muscle ring-finger protein-1 (MuRF1) and the 20S proteasome C2 subunit, were determined by real-time RT-PCR analysis using a CFX Connect^{TM} system (Bio-Rad Laboratories, Hercules, CA, USA). Isolation of tissue RNA and synthesis of cDNA were conducted as previously described (Yoshida *et al*., 2013), with minor modifications. The results are presented as ratios of the target mRNA to 18S ribosomal RNA (18S) levels, to correct for differences in the amounts of template cDNA used. The primer sequences used to amplify the target genes were as follows: atrogin-1 (sense 5′-CCA ACA ACC CAG AGA CCT GT-3′, antisense 5′-GGA GCT TCA CAC GAA CAT GA-3′ (Nakashima *et al*., 2006): NM_001030956.1), MuRF1 (sense 5′-TGT CTA CGG GCT GCA GAG GAA-3′, antisense 5′-GGT GCT CCC CCT TCT TGA GT-3′: XM_424369.2), the 20S proteasome C2 subunit (sense 5′-AAC ACA CGC TGT TCT GGT TG-3′, antisense 5′- CTG CGT TGG TAT CTG GGT TT-3′ (Nakashima *et al*., 2009): AF027978) and 18S (sense 5′-TAG ATA ACC TCG AGC CGA TCG-3′, antisense 5′-GAC TTG CCC TCC AAT GGA TCC-3′ (Abe *et al*., 2006): AF173612.1).

Statistical Analysis

All data are expressed as the mean±standard error (SE) of 4−9 individual samples. Statistical differences between the groups were identified using the Student's *t*-test or one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparisons test. $P<0.05$ was considered statistically significant.

Results

Effect of HS on Cellular Protein Levels and the Effect of the Proteasome Inhibitor in Avian Muscle Cells

To evaluate the involvement of the ubiquitin-proteasome protein degradation system in the decrease in protein levels due to HS treatment, cells were co-incubated with a specific inhibitor of the 20S proteasome during HS conditions. Whereas the protein content of the non-treated cells was significantly decreased by HS treatment compared to that of normal cells, this decrease was not observed in the proteasome inhibitor-treated cells (Fig. 1). In addition, the proteasome inhibitor had little effect on the protein content at normal temperatures. These results suggest that the ubiquitin-proteasome system may play a critical role in the decrease in cellular protein content that occurs due to HS treatment.

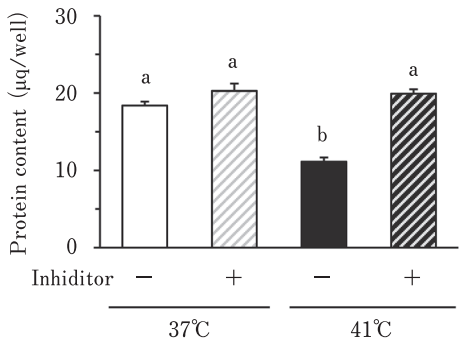

Fig. 1. **Effects of a 20S proteasome-specific inhibitor on the cellular protein content in 6 h hypothermia-exposed avian muscle cells.** The protein content was measured using the microBCA method. Values are means±SE, $n=9$. $^{a,b}P<0.05$, for each treatment; values with different letters are statistically different.

Time Course of Changes in Protein Content and mRNA Levels of Ubiquitin-proteasome Protein Degradation System-related Genes During HS Conditions

The time-dependent effects of HS treatment on the cellular protein and mRNA levels of ubiquitin-proteasome-related genes such as atrogin-1, MuRF1 and the proteasome C2 subunit were evaluated. As illustrated in Fig. 2A, there was no change in the protein content after 1 h of HS treatment, whereas a decrease was recorded after longer periods (3 h, 6 h). Little difference in the degree of the reduction in protein content was observed between the 3 h and 6 h HS-exposed cells. These results suggest that the decrease in protein content that occurred due to the 3 h HS treatment might be carried over into the protein levels observed at the 6 h time point. Atrogin-1 and MuRF1 are muscle-specific ubiquitin ligases (Bodine and Baehr, 2014). The mRNA levels of these atrogenes seem to be susceptible to proteolytic conditions such as fasting (Nakashima *et al*., 2006; Ohtsuka *et al*., 2011). As seen in Fig. 2, the mRNA levels of atrogin-1 (B) and MuRF1 (C) were significantly increased after 1 h of HS treatment, but subsequently diminished with longer periods of HS conditions. After 6 h of HS treatment, the atrogin-1 mRNA level was significantly lower than that in normal cells, and the MuRF1 mRNA level was restored to near-normal values. In addition, the mRNA level of the proteasome C2 subunit, which is one of the major subunits of the 20S proteasome that is a multicatalytic component of the 26S proteasome, did not vary at any stage of HS treatment (Fig. 2D). Overall the results suggest that protein ubiquitination may be augmented at the earlier stage of HS treatment, and that these proteins might subsequently undergo proteasomal degradation.

Changes in Intracellular ROS Production and Mitochondrial Superoxide Production During HS Conditions

The time course of changes in intercellular ROS production and mitochondrial superoxide production during HS conditions were evaluated to investigate if they changed in parallel with the changes in ubiquitin ligase mRNA levels. As illustrated in Fig. 3A, intracellular ROS production was not affected by the 1 h HS treatment, but was significantly increased by the 3 h and 6 h treatments compared to normal cells. In contrast, mitochondrial superoxide production was significantly increased by the 1 h HS treatment, with an increase still being observed at the 6 h time point (Fig. 3B).

Scavenging Mitochondrial Superoxide Reduces Atrogenes mRNA Levels in HS-exposed Cells

Given our finding of simultaneous increases in mitochon-

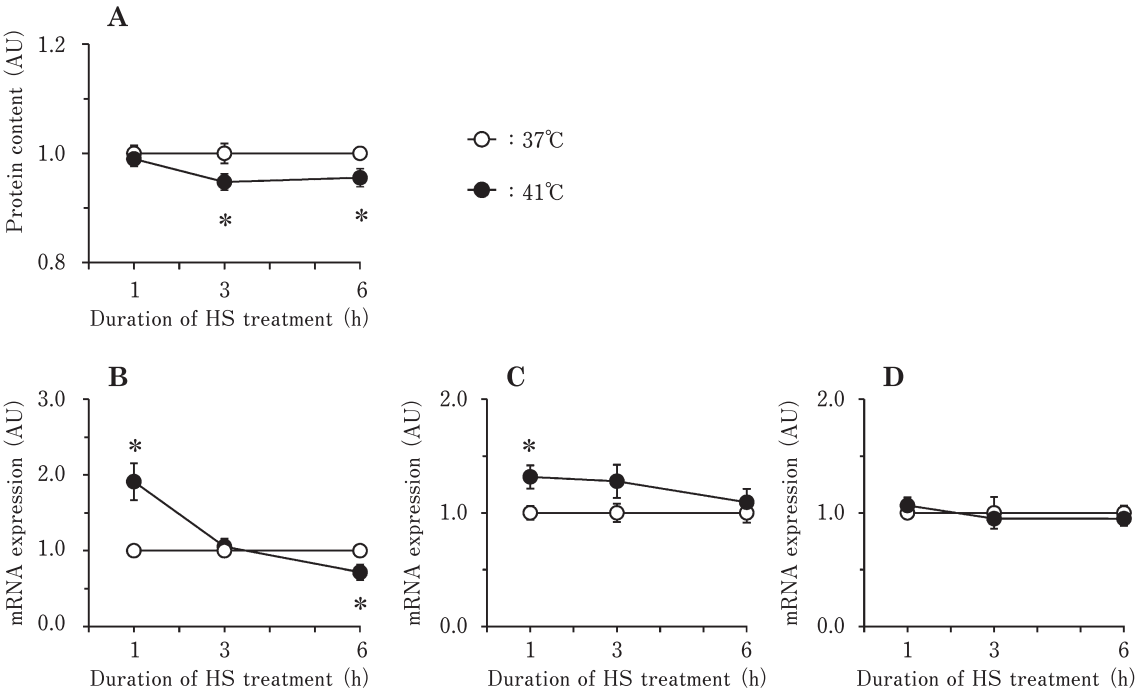

Fig. 2. **Time-dependent effects of on cellular protein content (A), and the mRNA levels of atrogin-1 (B), MuRF1 (C) and the 20S proteasome C2 subunit (D) in avian muscle cells.** Real-time RT-PCR was used to quantify the mRNA levels, and the results were normalized to 18S transcript levels. The values were expressed as fold change relative to the control values at each time point. Values are means±SE, n=4–8. *P<0.05 compared to the control values at each time point.

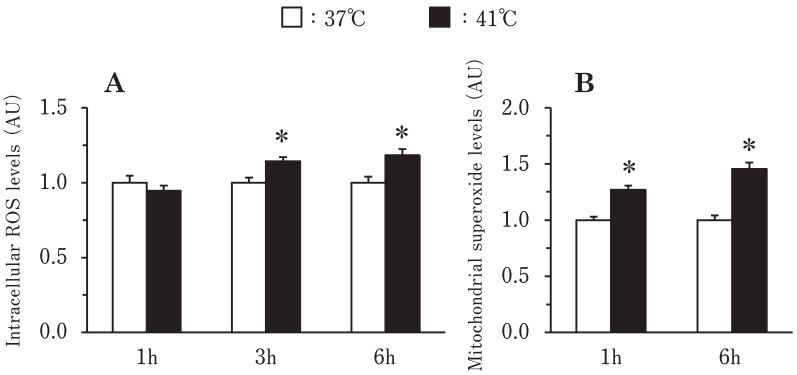

Fig. 3. **Time-dependent effects of HS on intracellular ROS production (A) and mitochondrial superoxide production (B) in avian muscle cells.** Intracellular ROS production and mitochondrial superoxide production were fluorometrically determined using CM-H_2DCFDA and MitoSOX^{TM} Red, respectively, with the results being corrected for protein levels. Values are means±SE, n=7–8. *P<0.05 compared to the control values at each time point.

drial superoxide production (Fig. 3B) and the atrogenes (atrogin-1 and MuRF1) mRNA levels in the 1 h HS-exposed cells (Fig. 2B–2C), we hypothesized that mitochondrial superoxide production could play an influential role in inducing the transcription of these genes in HS-exposed cells. To verify this, Tmp was added to muscle cells prior to HS

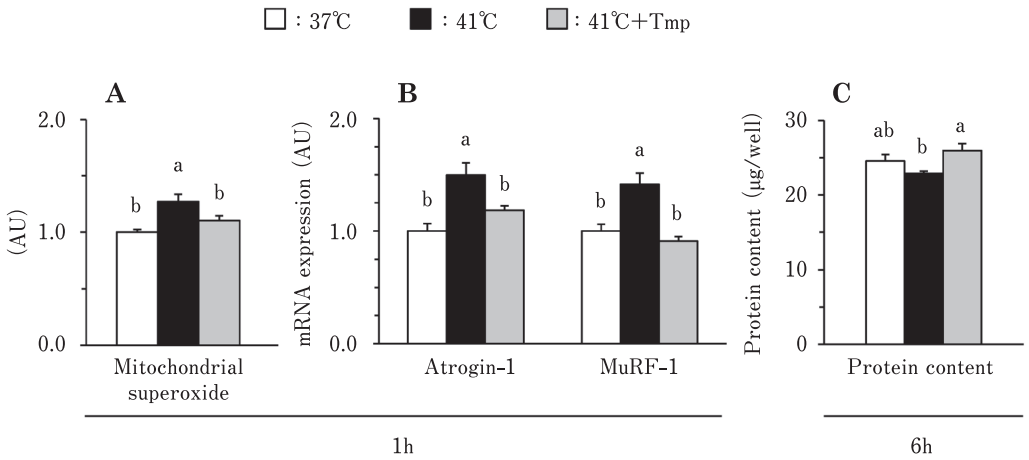

Fig. 4. **Suppressive effects of Tmp on mitochondrial superoxide production (A), mRNA levels of ubiquitin ligases (B) and cellular protein content (C) in 1 h or 6 h HS-exposed avian muscle cells.** Mitochondrial superoxide radical levels were fluorometrically determined using MitoSOX™ Red, with the results being corrected for protein levels. Real-time RT-PCR was used to quantify the mRNA levels, and the results were normalized to 18S transcript levels. Values are means±SE, $n=5$–6. $^{a,b}P<0.05$, for each treatment; values with different letters are statistically different.

treatment. As a result, mitochondrial superoxide production in Tmp-pretreated cells following 1 h of HS treatment was restored to near-normal values (Fig. 4A), and the atrogin-1 and MuRF1 mRNA levels in these cells were also significantly decreased (Fig. 4B). Moreover, following the longer HS treatment of these cells (6 h), the protein content was also restored to near-normal values (Fig. 4C). Overall these results suggest that mitochondrial superoxide production may play an important role in initiating protein ubiquitination via atrogin-1 and MuRF1 transcription at an early stage of HS treatment, and that proteasomal degradation might subsequently occur.

Discussion

In agreement with previous studies (Luo *et al*., 2000; Nakashima *et al*., 2004a), the present study has shown that HS stimulates the ubiquitin-proteasome system, which leads to protein degradation in skeletal muscle cells. It has also provided the first evidence that mitochondrial superoxide production may play an important role in evoking protein ubiquitination, thereby promoting proteasomal degradation in these cells. Several studies has reported that ROS (in many case, H_2O_2) may be associated with muscle protein catabolism via ubiquitin-conjugating activity and calpain-1 (Li *et al*., 2003b; McClung *et al*., 2009), and growing evidence suggests that mitochondria-derived ROS promote muscle atrophy and autophagy in skeletal muscle (Gilliam *et al*., 2012; Rahman *et al*., 2014). Thus, HS-induced mitochondrial superoxide production is also likely to play a pivotal role in initiating protein degradation.

The signal transduction mechanisms by which mitochondrial superoxide production regulates transcription of atrogin-1 and MuRF1 genes should be considered. These atrogenes transcription are regulated by transduction pathways via nuclear factor-kappa B (NF-κB) or forkhead box O (FoxO) transcription factors (Bonetto *et al*., 2009). Li *et al*. (2003b) have reported that H_2O_2 is a weak activator of NF-κB in C2C12 myotubes. FoxO activation (dephosphorylation) induces muscle atrophy via increases in the expression of these atrogenes (Sandri *et al*., 2004). Furthermore, protein kinase B (PkB, also known as Akt) is one of the inhibitory factors that regulates FoxO via phosphorylation (Brunet *et al*., 1999), and it has been reported that acute nutrition deprivation stimulates mitochondrial superoxide production, which inhibits Akt, thereby dephosphorylating (activating) FoxO3a (Rahman *et al*., 2014). Based on these lines of evidence, one or some of the above factors might implicate in the induction of atrogin-1 and MuRF1 transcription under HS conditions. However, there is no information regarding on changes in the above factors in response to HS treatment as far as we know. When this is established, we will be in a position to better understand the mechanism that initiates the ubiquitination process under HS conditions.

Given that superoxide is not able to permeate the mitochondrial membrane, one issue that needs to be considered is at which mitochondrial sites superoxide is generated, thereby leading to ubiquitin ligase gene transcription in HS-exposed cells. Our previous studies using isolated mitochondria from HS-exposed birds showed that the overproduction of superoxide mainly occurred at complex I due to reverse electron flow (Kikusato and Toyomizu, 2013a). In this context, it is

well known that complex I-dependent superoxide is released into the mitochondrial matrix (St-Pierre *et al*., 2002; Miwa *et al*., 2003), which allows us to assume that superoxide generated from different sources might be involved in the induction of atrogin-1 and MuRF1 transcription in HS-exposed cells. Mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) is one of the major sources of superoxide production in mitochondria (Drahota *et al*., 2002; Miwa *et al*., 2003; Tretter *et al*., 2007), and ROS generated by this enzyme are equally released to both sides of the mitochondrial inner membrane (Miwa and Brand, 2005; Orr *et al*., 2012). In this regard, we have recently obtained evidence that HS stimulates superoxide production via mGPDH in isolated muscle mitochondria (Kikusato and Toyomizu, 2013b). It is therefore conceivable that superoxide generated from mGPDH might induce the transcription of ubiquitin ligase genes, thereby activating the ubiquitination process under HS conditions.

Whereas mitochondrial superoxide production may play an influential role in inducing transcription of the atrogenes in 1 h HS-exposed cells, these up-regulation did not occur in 6 h HS-exposed cells in spite of the increase in the mitochondrial superoxide levels (Fig. 2B, 2C and Fig. 3B). It can be assumed that an increase in Cu/Zn-superoxide dismutase (SOD1) or cytochrome *c* might be involved in the discrepancy observed in these cells. They are unable to eliminate superoxide radical released into the mitochondrial matrix, but are able to the radical released into the intermembrane space (Pereverzev *et al*., 2003; Iñarrea *et al*., 2005). From these findings, it was suggested that SOD1 or cytochrome *c* expression might be increased in 6 h HS-exposed cells, thereby abolishing the effect of mitochondrial superoxide on the atrogenes transcription. However, our previous study showed that the mRNA levels of SOD1 were not changed in 6 h HS-exposed cells (Yoshida *et al*., 2013). Thus, we infer that an increase in cytochrome *c* activity might disable the effect of mitochondrial superoxide in such conditions.

The ubiquitin-proteasome protein degradation system comprises ubiquitination and proteasomal degradation processes. The present study showed that the treatment of HS-exposed cells with a proteasome inhibitor resulted in a suppression of the decrease in protein content (Fig. 1). From this experimental fact, we considered that proteasomal degradation process would also be induced by exposure to HS. However, the present study showed that proteasome C2 subunit mRNA levels were not altered in HS-exposed cells (Fig. 2D). Therefore, it has to be considered whether proteasomal degradation was increased in response to HS and how cellular protein was decomposed by the ubiquitin-proteasome system in HS-exposed cells. We proposed two possible explanations to these questions. Firstly, proteasomal degradation in HS-exposed cells may not be changed in accordance with proteasome C2 subunit mRNA levels (Fig. 2D), and therefore only an ubiquitination may be the rate-limiting reaction for inducing protein degradation in these cells. Recent researches have suggested that the transcription of proteasome seems to also be induced when the proteolysis activity is increased (Kobayashi *et al*., 2004; Itoh *et al*., 2004; Taguchi *et al*., 2011). Several proteasome subunits is induced through nuclear factor E2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) pathway. In this pathway, Nrf2 is constantly degraded via the ubiquitin–proteasome pathway in a Keap1-dependent manner under normal conditions, while this degradation ceases in the presence of electrophiles or ROS. Accumulated Nrf2 then activates target genes for cytoprotection including proteasome subunits. It can be therefore assumed that these transcriptional events might not occur in HS-exposed cells. Secondly, proteasomal proteolysis may be induced independent of the above transcription pathway. This idea is supported by the report of Morita *et al*. (1996). They showed that hyperthermic treatment of C2C12 myotubes resulted in an increase in the 26S proteasome activity but not in the mRNA levels. In view of this finding, it is possible that proteasomal proteolysis may also be increased without an increase in the transcription in response to HS, though the precise machinery inducing proteasomal activity remains unclear. Thus, in order to determine whether proteasomal degradation is enhanced due to HS treatment, Nrf2/Keap1 pathway and/or proteasomal activity are required to be investigated.

In order to give a new insight into HS-induced protein catabolism *in vivo*, it is also important to consider an endocrine factor affecting transcription of atrogin-1 and MuRF1 genes, because it is a major difference between *in vivo* and *in vitro* experiments. Plasma corticosterone levels were increased in response to HS treatment (Yunianto *et al*., 1997; Willemsen *et al*., 2011), and this glucocorticoide seems to increase mRNA levels of the atrogenes in cultured myotubes (Menconi *et al*., 2008). Moreover, it has been reported that circulating levels of tumor necrosis factor-α (TNF-α) and its muscular mRNA levels were also increased in response to HS treatment (Welc *et al*., 2012). TNF-α is known to stimulate mitochondrial superoxide production (Li *et al*., 1999; Corda *et al*., 2001; Kastl *et al*., 2014), and is able to activate NF-κB signal transduction, which is one of pathways responsible for inducing the atrogenes transcription as above mentioned. From these lines of findings, it is highly likely that there is more complicated mechanisms that induces protein catabolism in birds reared under HS conditions. For future research, it is important to elucidate the role of mitochondrial superoxide production in the intracellular and extracellular mechanisms occurred in heat stressed-birds.

In the present study, avian muscle cells were subjected to 41℃ conditions, which is approximately same as body temperature of domestic chickens. Therefore, it can be suggested that hyperthermic treatment with higher than 41℃ should be conducted to investigate avian species-specific protein catabolism. Our previous study reported that little difference in decreases in protein levels was observed between 41℃ and 43℃ HS-exposed in the presence of FBS (Yoshida *et al*., 2013), which allowed us to assume that protein catabolism might be temperature-independent in

avian muscle cells. However, we consider that more detailed investigation is required in order to determine the avian protein catabolism in response to hyperthermic conditions.

In conclusion, the present study provides insight into the mechanism that regulates the intracellular protein degradation system in HS-exposed muscle cells: overproduction of mitochondrial superoxide may play an important role in initiating the ubiquitin-proteasome system.

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