ニワトリ骨格筋におけるミトコンドリアの酸化的リン酸化および活性酸素産生の代謝調節能

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Roles of mitochondrial oxidative phosphorylation and reactive oxygen species generation in the metabolic modification of avian skeletal muscle

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Abstract

Mitochondria are double membrane-bound organelles found in most eukaryotic cells and serve as the centers of intracellular metabolism. One of their major functions is ATP production by oxidative phosphorylation (OXPHOS). OXPHOS is an energy transduction system that is based on coupling NADH/$FADH_2$ oxidation-driven electron transfer with ADP phosphorylation, with the inner membrane potential ($\Delta\Psi$) serving as a mediator. The coupling efficiency of OXPHOS contributes to the growth rate of chickens. The mechanistic understanding of this efficiency is therefore quite important. Herein, we review the general mechanisms underlying OXPHOS, and describe modular kinetic analysis, which is the methodology used for determining OXPHOS efficiency. Moreover, this review describes our experimental adaptation of the kinetic method to avian muscle mitochondria. We show the analysis for the differences in the coupling efficiency between meat- and laying-type chickens and the implication of these differences in determining the growth rate in these birds. The review also discusses the role of mitochondria as a major intracellular reactive oxygen species (ROS) generator and their effects on cellular metabolism. Mitochondria-generated ROS cause oxidative disturbance in cells, and participate in intracellular signal transduction pathways evoking cell death and protein catabolism, in response to several physiological and pathological stimuli. We describe that heat stress (HS) stimulates mitochondrial ROS generation, which may be caused by OXPHOS alterations and the subsequent increase in $\Delta\Psi$. Finally, we suggest that HS-induced mitochondrial ROS generation may play an important role in the induction of ubiquitin-proteasome-dependent protein degradation in avian skeletal muscle, which is partially responsible for growth retardation in the HS-treated birds.

1. Introduction

Mitochondria are double membrane-bound organelles found in most eukaryotic cells. The morphology and functions of this organelle transmute along with intracellular metabolic alterations. A major function of mitochondria is ATP production through oxidative phosphorylation (OXPHOS). In addition to this role, the mitochondria are also central to apoptosis[1], Ca^{2+} regulation[2], adaptive thermogenesis[3-5], and signal transduction via reactive oxygen species (ROS) generation[6,7]. Proper mitochondrial function is therefore critical to the cell[8].

For the past dozen years or so, we have investigated the metabolic role of avian muscle mitochondria in thermogenesis induced by exposure to low temperatures[9,10], as well as their role in ROS generation under heat stress (HS) conditions[11-13] in chickens. In this review, we initially describe the general mechanism of ATP production in OXPHOS, and the methodology used to study this mechanism. Furthermore, we provide

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information on studies on mitochondrial bioenergetic functions, especially regarding the coupling efficiency of OXPHOS and the HS-induced ROS generation. The review describes the effects of the latter on intracellular oxidative disturbance and protein degradation in avian skeletal muscle.

2. Mitochondria are the powerhouses of cells

Mitochondrial OXPHOS is the powerful and highly organized energy production system through which 36 moles of ATP are produced from 1 mole of glucose. Reducing equivalents, NADH and $FADH_2$, are produced by the TCA cycle (tricarboxylic acid cycle, also known as citric acid cycle, or Krebs cycle) or through β-oxidation. NADH and $FADH_2$ are oxidized at the respiratory complexes I (NADH: ubiquinone oxidoreductase, EC 1.6.5.3) and II (succinate dehydrogenase, EC 1.3.5.1), respectively (Fig. 1). The produced electrons are sequentially transferred from complexes I and II to ubiquinone (UQ), complex-III (coenzyme Q: cytochrome *c*-oxidoreductase, EC 1.10.2.2) and cytochrome *c* (Cyt *c*), finally reaching complex-IV (cytochrome *c* oxidase, EC 1.9.3.1) where they react with oxygen molecules. During the substrate oxidation and the consequent electron transports, protons are pumped from the matrix into the intermembrane space through the inner membrane by complexes I, III, and IV, forming a difference in potential ($\Delta\Psi$) across the inner membrane. $\Delta\Psi$ is used by the ATP synthase (ATPase) to generate ATP from ADP and monophosphate (Pi). Thus, mitochondrial OXPHOS is an energy transduction system that is based on the coupled reactions of the substrate oxidation-driven electron transfer and ADP phosphorylation, which are connected with $\Delta\Psi$ serving as an intermediate.

However, not all energy generated by the transfer of electrons is used for the production of ATP. An amount of pumped protons leak back to the matrix due to defects affecting the integrity of the inner membrane, reducing the $\Delta\Psi$. This unavoidable electrical dissipation is called the basal proton leak in mitochondria bioenergetics (Fig. 1); it constitutes one of factors determining the coupling efficiency of ATP production in OXPHOS.

In addition to the unavoidable leaks caused by membrane defects, cells may actually induce proton leaks by expressing proteins that reduce coupling efficiency, and are called UCPs (uncoupling proteins). UCP1, which

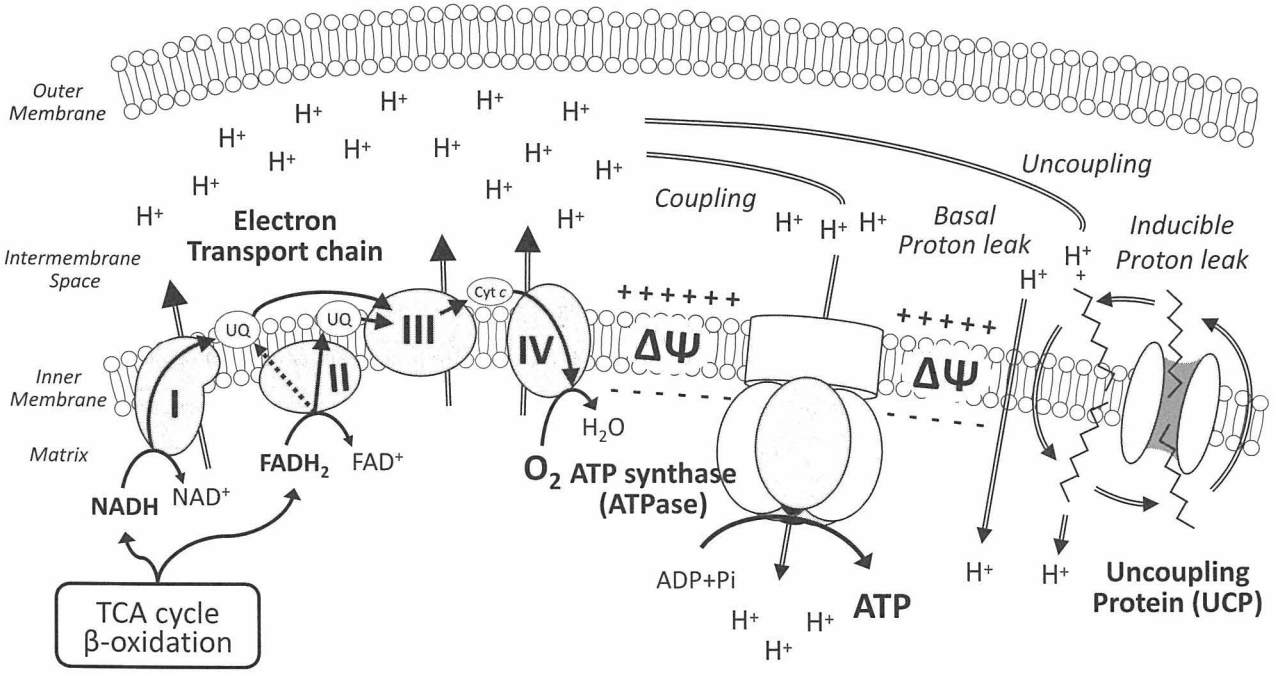

Fig. 1. Schematic representation of mitochondrial oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) formation. UQ: Ubiquinone, Cyt *c*: Cytochrome *c*, $\Delta\Psi$: Membrane potential.

is expressed in brown adipose tissue, is known to act as a thermogenic protein in cold environments through the activation of inducible proton conductance. Two UCP1 isoforms, the ubiquitously expressed UCP2, and UCP3 which is predominantly expressed in skeletal muscle tissue[14-17], induce proton leaks after being activated by free fatty acids[18], superoxide[19], lipid hydroperoxides[20] and 4-hydroxy-2-nonenal[21]. An avian uncoupling protein (avUCP), which shares a 71–73% amino acid identity with both mammalian UCP2 and UCP3, was identified in chicken skeletal muscle[10,22].

3. Coupling efficiency of mitochondrial OXPHOS

Mitochondrial OXPHOS is systematically organized as described above, and it is important to estimate the coupling efficiency of the energy transduction system. The coupling efficiency of OXPHOS can be defined as the proportion of the mitochondrial respiratory rate that is used to drive ATP synthesis[23]. To precisely calculate this proportion, Brand and his co-workers established a novel kinetic method, referred to as modular kinetic analysis, that is based on the simultaneous measurement of the mitochondrial inner-membrane $\Delta\Psi$ and of the oxygen consumption rate[24].

Modular kinetic analysis divides OXPHOS into the following three modules (Fig. 2A): (*i*) "substrate oxidation", which includes the $\Delta\Psi$-producing reactions (substrate oxidation followed by electron transfer), (*ii*) "phosphorylation", where a part of the $\Delta\Psi$ is consumed for ATP synthesis, and (*iii*) "proton leak", which includes proton leaks (basal and UCP-mediated) that consume $\Delta\Psi$ without producing ATP[25]. The models representing each kinetic reaction are illustrated in Fig. 2B. The proportion of mitochondrial respiratory rate driving ATP synthesis is calculated by subtracting the portion of non-phosphorylation respiration rate from the overall respiration rate, and then dividing by the overall respiration rate. Respiratory control ratio (RCR), which

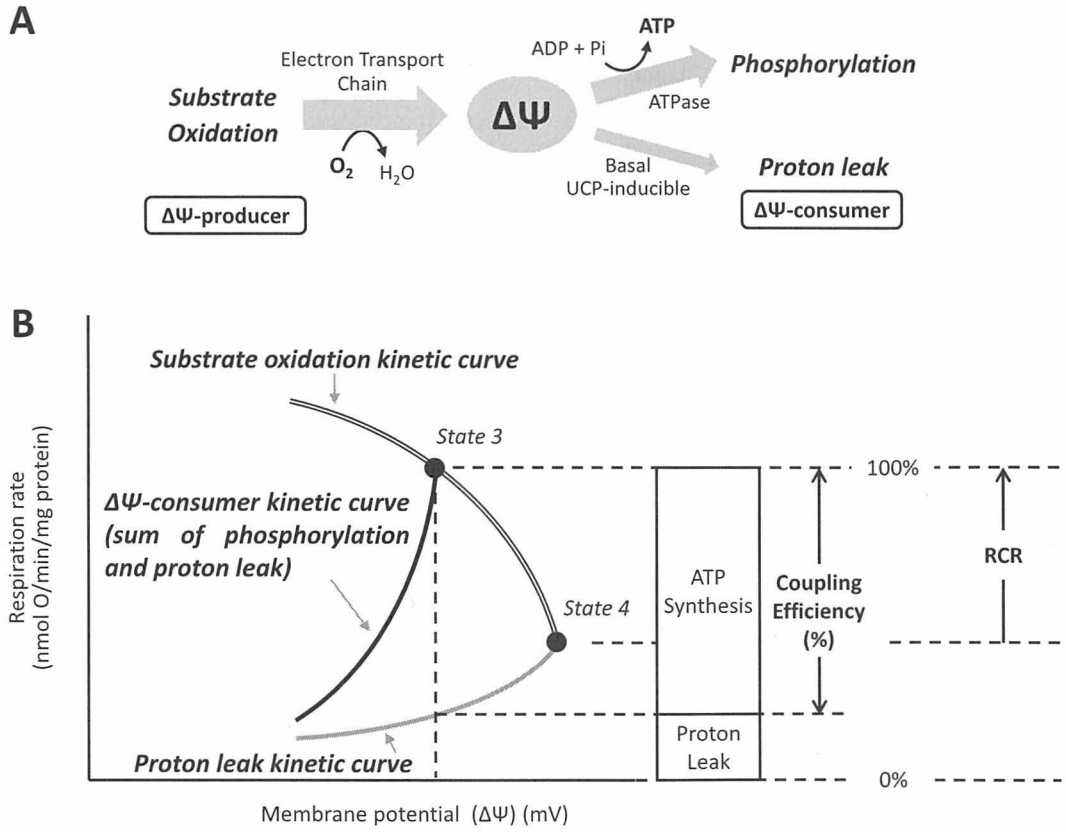

Fig. 2. Schematic representations of the three-branch modular system of mitochondrial oxidative phosphorylation (OXPHOS) (A), and of the kinetic curves comprising the modular kinetic analysis, as well as the values involved in calculating the percentage representing coupling efficiency (B).

is the ratio of the maximal respiration rate consumed for ATP synthesis (state 3) to the non-phosphorylation respiration rate (state 4), is a classical way to evaluate coupling efficiency. However, this approach will slightly underestimate the true coupling efficiency. As proton leak also occurs during phosphorylation, the proton leak-dependent respiration rate is actually smaller than the state 4 respiration rate (Fig. 2B). Modular kinetic analysis can provide data for the respiration rate due to proton leak in ATP synthesis by measuring $\Delta\Psi$.

4. Difference in the coupling efficiency of OXPHOS between meat and laying-type chickens

Meat- and laying-type chickens have undergone extensive selective breeding for maximizing meat and egg production, respectively, resulting in a faster growth rate in meat-type chickens compared with laying-type birds. A lower protein degradation rate has been proposed as one of the mechanisms causing the growth difference between the two types of chickens[26-28]. Mitochondrial basal proton leak is a significant contributor to the standard metabolic rate of the whole animal[29]. In mammals, a negative correlation between the proton leak rate and body mass has been observed[30]. Furthermore, it has been reported that the feed intake versus metabolic body mass index is higher in laying-type chickens than in meat-type chickens, indicating the existence of either a lower metabolic efficiency or a higher energy expenditure in laying-type chickens[31]. Our investigation using modular kinetic analysis demonstrated that skeletal muscle (*M. pectoralis*) mitochondria from laying-type chickens displayed a higher basal proton leak rate, and a lower $\Delta\Psi$ -consuming reaction compared with meat-type chickens, both of which result in a lower coupling efficiency of OXPHOS in laying-type chickens[32]. We also found that the avUCP expression level was higher in laying-type chickens than in meat-type chickens. Our findings suggest that mitochondrial coupling efficiency in skeletal muscle may be a major contributor to determining the body mass of chickens.

5. Mitochondrial ROS generation and heat stress in poultry

ROS are produced continuously as a byproduct of aerobic metabolism, with mitochondria being their primary source. Oxygen free radicals are reactive molecules, but can be converted into hydrogen peroxide (H_2O_2) by the manganese-superoxide dismutase (Mn-SOD, also known as SOD2); hydrogen peroxide is subsequently detoxified in mitochondria by the glutathione peroxidase (GPx). However, this anti-oxidative protection is not perfect; superoxide or H_2O_2 that evade the system can be converted into highly reactive hydroxyl radicals ($^{\bullet}OH$) or hydroperoxyl radicals ($HOO^{\bullet}$) (Fig. 3), which are able to cause irreversible molecular damage, termed as oxidative stress.

Oxidative stress occurs due to several physiological and pathological stimuli, and heat exposure is one of the environmental stressors causing oxidative damage[33,34]. As hyperthermia results in growth retardation[35] and meat quality loss[36,37] in poultry production, the mechanistic understanding of HS-induced oxidative disturbance and its effect on the intracellular metabolism are of great importance for poultry farming. Our previous investigations have revealed that the reduction in growth performance in HS-treated birds is associated with an increase in oxidative damage to skeletal muscle[35], and that overproduction of mitochondrial ROS may play a pivotal role in the induction of oxidative damage in HS-treated birds[11,38].

In mitochondria, superoxide ($^{\bullet}O_2^-$) is generated as the primary ROS via electron leakage followed by the one-electron reduction of oxygen molecules. Eleven distinct mitochondrial sites, associated with substrate oxidation and OXPHOS, have been identified as sites of superoxide production[39]. The electron transport chain complexes I and III are two of these sites[40]. Superoxide production at the two complexes is closely related to the electron flow and the magnitude of $\Delta\Psi$[41-44]. Forward electron flow toward the complex IV produces a relatively small amount of superoxide at complexes I and III, while a bigger amount is produced at complex I by the reverse

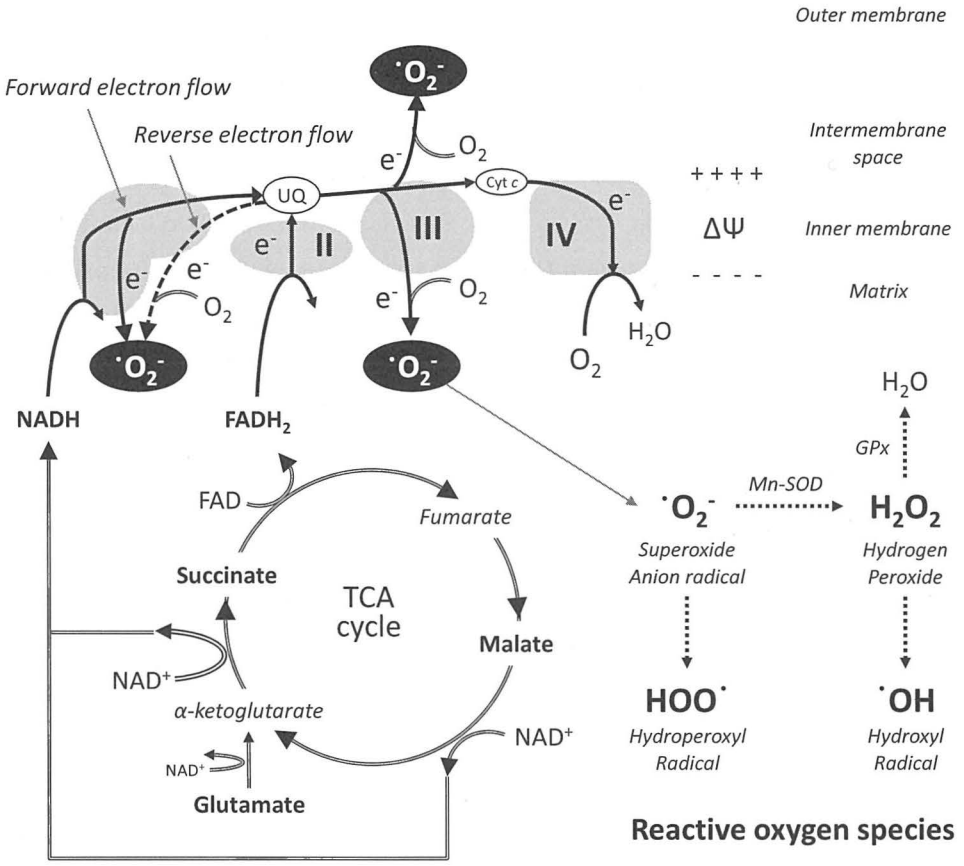

Fig. 3. Mitochondrial superoxide generation and subsequent reactive oxygen species (ROS) formation.

electron flow from complex II (Fig. 3). Both of these amounts increase as ΔΨ increases[45]. Our investigation demonstrated that the mitochondrial ROS production rate and the ΔΨ values in HS-treated birds were higher compared to those of birds kept at a thermoneutral environment[46], which led us to seek the mechanism through how HS-treatment leads to an increase in ΔΨ.

6. The mechanism of ΔΨ increase in response to HS treatment

The magnitude of ΔΨ is tightly regulated, and determined by the balance between the ΔΨ-producer and the two ΔΨ-consumers, as illustrated in Fig. 2A. Our previous investigation showed that the activity of substrate oxidation was markedly increased in response to HS treatment[46]. Furthermore, we found that the expression levels of the ΔΨ-dissipating avUCP were decreased in the muscle mitochondria of HS-treated birds, with respect to both mRNA and protein abundance[47], leading to a down-regulation of the avUCP-induced proton leak. These results suggest that the HS-induced increase in ΔΨ results from both an increase in ΔΨ-producing reactions, as well as from a decrease in ΔΨ-consuming reactions. Regarding the role of muscle avUCP in regulating the HS-induced increase in ΔΨ, we showed that laying-type chickens, whose muscle avUCP expression level is higher than that of meat-type chickens[32], exhibited resistance to HS-induced mitochondrial ROS production and oxidative damage[48]. From these lines of evidence, we postulate that the regulation of the avUCP expression level is an effective means of attenuating HS-induced oxidative disturbance.

7. Nutritional regulation of avUCP to counteract HS-induced oxidative disturbance

Several dietary factors such as theaflavins[49], epigallocatechin gallate[50], fish oil[51], fucoxanthin[52], olive oil[53,54], and oleuropein[55] (which is a phenolic

compound isolated from olive oil), have been reported to upregulate the cellular UCP level through distinct signaling pathways. Our previous studies have shown that meat-type chickens fed an olive oil-supplemented diet exhibited a suppression of HS-induced mitochondrial ROS generation[56], while co-supplementation with olive oil and oleuropein alleviated the ROS production in HS-treated birds via a mechanism dependent on the avUCP expression (Kikusato *et al.*, *unpublished data*). The upregulation of avUCP expression due to oleuropein may be mediated by the activation of the peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α)[57], which is a transcriptional co-factor of UCPs[58]. Considering that avUCP is a mitochondrial protein that dissipates energy that would otherwise be used for ATP generation, it can be proposed that the upregulation of this metabolic efficiency-reducing protein may result in a reduction in growth efficiency. We recently obtained data contradicting this hypothesis, as we observed that a low-concentration oleuropein supplementation (<2.5 ppm) did not affect growth performance and feed efficiency, but was able to suppress muscle oxidative damage in normal feeding conditions (Kikusato *et al.*, *unpublished data*). Further investigation is required to determine additive amounts that are both effective in suppressing HS-induced oxidative stress, and financially efficient.

8. Involvement of mitochondria-derived ROS in HS-induced muscle protein degradation

A decrease in skeletal muscle mass and the accumulation of oxidative damage are two of the physiological effects taking place under hyperthermic conditions. Taking into account recent findings according to which mitochondria-produced ROS induce atrophy[59,60], apoptosis[61,62], and autophagy[63], it can be assumed that the ROS generated due to HS exposure may function as inducers of protein degradation in avian muscle cells. It is accepted that corticosterone induces the protein degradation observed in HS-treated birds[64], and several studies have demonstrated that treatment with corticosterone or its synthetic analog, dexamethasone, induce muscle protein degradation by upregulating the transcription of muscle specific atrogenes, such as those encoding atrogin-1 and MuRF1[65,66], both of which act as ubiquitin ligases in the ubiquitin-proteasome intracellular protein degradation system (UPS). Contrary to the corticosterone hypothesis, it has been found that hyperthermic treatment of cultured muscle cells results in excess mitochondrial ROS production[67] and enhanced muscle protein degradation, obviously through a mechanism that can't be dependent on corticosterone secretion[68]. Based on this evidence, it is reasonable to assume that mitochondria-derived ROS may induce protein catabolism in skeletal muscle in response to HS treatment. This hypothesis was substantiated by our animal and cell culture studies. In HS-treated birds, circulating levels of corticosterone, muscle UPS-related genes expression, and mitochondrial ROS generation, increase at the early stages of treatment[69]. Thereafter, we evaluated the effects of HS-induced mitochondria-derived ROS and physiologically relevant levels of corticosterone (0 to 30 ng/ml) on the onset of protein degradation in cell cultures. Our results suggest that the mitochondria-derived ROS, rather than corticosterone, play the central role in instigating muscle proteolysis in HS-treated birds[70] (Fig.4) These studies provide novel findings indicating that mitochondria-produced superoxide in avian HS-treated muscle cells may contribute not only to oxidative damage, but also to intracellular protein degradation, which in turn suggests that the regulation of mitochondrial ROS generation is extremely important to protein homeostasis.

9. Mitochondrial functional specialization in different muscle fiber types

In our aforementioned studies, *M. pectoralis* muscle was the main model used for evaluating avian mitochondrial bioenergetics. Skeletal muscles are composed of three muscle fiber types that are referred to as fast-twitch glycolytic (type IIB), fast-twitch oxidative (type IIA), and slow-twitch oxidative (type I). The three types differ in their mitochondrial content, and this difference in

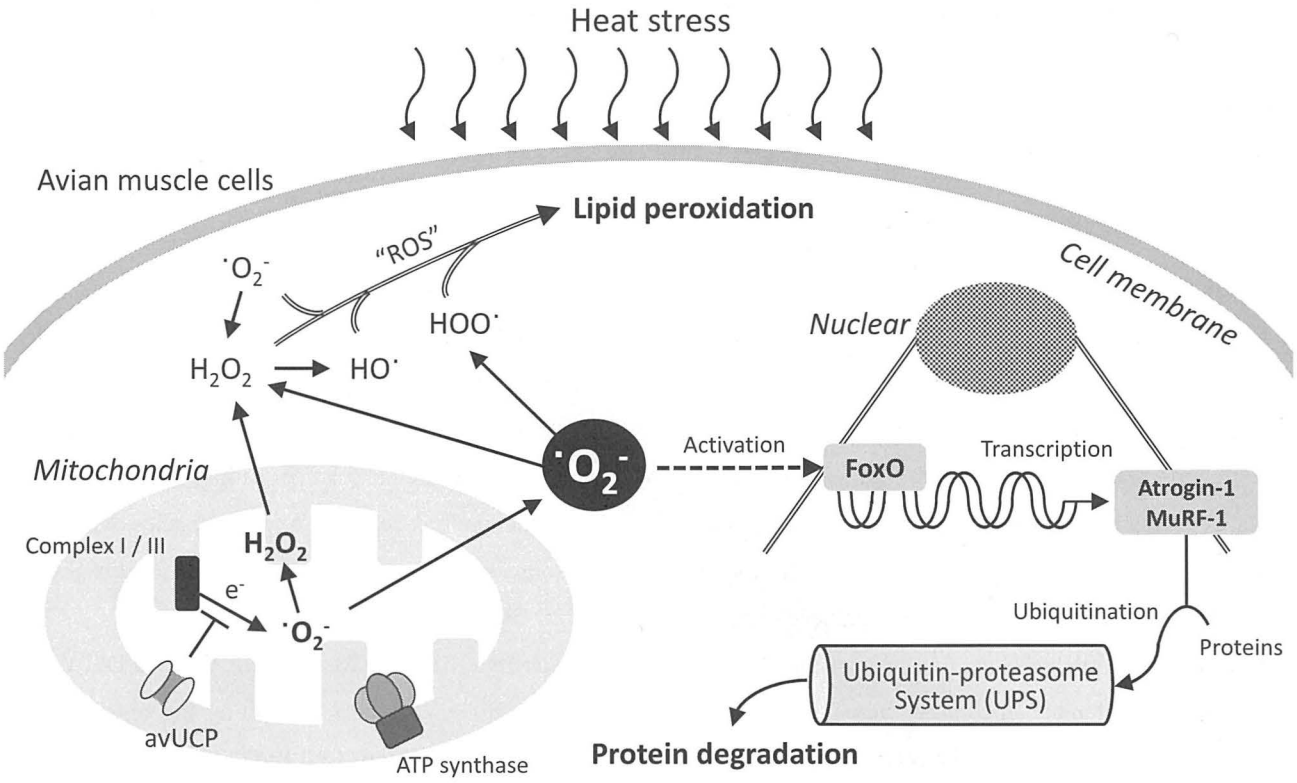

Fig. 4. Involvement of heat stress-induced mitochondrial superoxide generation in inductions of lipid peroxidation and ubiquitin-proteasome protein degradation system in avian muscle cells.

mitochondrial quantity was for a long time considered their major difference. Today, it is known that muscles with different muscle fiber compositions have different bioenergetic characteristics[71]. In regard with the metabolic differences observed in mitochondria isolated from several types of muscle in broiler chickens, we have found that mitochondria isolated from muscles composed of type I (*M. pubo-ischio-femoralis pars medialis*) or type IIA fibers (*M. pubo-ischio-femoralis pars lateralis*) exhibited a higher phosphorylation respiration rate compared with that of muscles made of type IIB fibers (*M. pectoralis*) (Hakamata *et al.*, *unpublished data*).

Regarding the HS effects on several types of avian skeletal muscle, we found that hyperthermic treatment induced mitochondrial ROS generation and oxidative damage in *M. pectoralis* (type IIB: 100%,[9]) but these increases did not occur in the *gastrocnemius* muscle (Kikusato *et al.*, *unpublished data*), which contains approximately 43% type-IIB fibers[72]. The mRNA levels of avUCP in *M. pectoralis* were downregulated in response to HS treatment, but this decrease was not observed in the *gastrocnemius* muscle. Moreover, the level of Mn-SOD mRNA in the *M. pectoralis* muscle did not change in response to HS treatment, while the level in the *gastrocnemius* muscle was augmented by HS treatment. These results suggest that avian skeletal muscles containing a high percentage of type-IIB fibers could be less tolerant to HS-induced oxidative disturbance than muscles with lower percentages of this type, and that the transcription regulation of the avUCP and Mn-SOD genes in muscles may play an important role in the induction of oxidative disturbance due to HS treatment. This study also demonstrated that the expression levels of avUCP, MnSOD, and PGC-1α (which expresses a co-factor for the transcriptional regulation of MnSOD expression) in the *gastrocnemius* muscle are higher compared to those of *M. pectoralis* under thermoneutral conditions (Kikusato *et al.*, *unpublished data*), which might contribute to the observed transcriptional differences in response to HS treatment. In order to elucidate the regulation mechanisms of HS-induced oxidative disturbance, further comparisons of the

molecular machinery governing mRNA expression in the different muscles are required. This may open the way for new approaches, such as line selection method of chicken with avian skeletal muscles containing a lower percentage of type-IIB fibers.

10. Conclusions

Herein, we reviewed the difference in coupling efficiency of mitochondrial OXPHOS between chickens exhibiting different growth rates, and the central role of mitochondrial ROS generation in the induction of oxidative stress and protein degradation in avian skeletal muscles. This review also provides further insight into the molecular machinery that regulates HS-induced oxidative disturbance. Given that skeletal muscle tissue in chickens, as in large mammals including humans, plays an important role in overall bioenergetics and homeostasis, the current results obtained from chicken are likely to find application in research on large mammals and general health. Overall, the findings suggest the importance of mitochondrial functions in avian skeletal muscle; further understanding of mitochondrial bioenergetics has the potential to contribute to the enhancement of poultry production, and gives significance to research in mitochondrial bioenergetics, physiological stress-nutritional factor-gene interaction study, and oxidative stress management.

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