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SHORT COMMUNICATION

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Differential effects of heat stress on oxidative status of skeletal muscle with different muscle fibre compositions in broiler chicken

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ABSTRACT. Skeletal muscles are composed of two major muscle fibre types, glycolytic and oxidative, which can be differentiated using their mitochondrial content. Mitochondria are a major generator of reactive oxygen species, and muscles have adapted them to possess oxidative resistance to counteract the oxidative damage. The present study aims to clarify the oxidative tolerance of heat stress (HS) in different types of skeletal muscles of broiler chickens. Exposure of 3-week-old broiler chickens to HS conditions (34 °C, 12 h) resulted in significantly higher lipid peroxidation in *Musculus pectoralis* (*Pec*), which consists entirely of glycolytic muscle fibres (type IIB), than in thermoneutral (TN) birds. This increase did not occur in *gastrocnemius* (*Gas*) muscle, which has a lower proportion of type IIB fibres (65–80%). HS treatment resulted in significantly higher mitochondrial H_2O_2 production in *Pec* muscle but not in *Gas* muscle. In both muscles, HS treatment did not alter the gene expression levels of cytosolic antioxidative enzymes, superoxide dismutase (SOD) 1, catalase and glutathione peroxidase-4. In *Pec* muscle, there was no difference in SOD2 mRNA levels between TN and HS birds, while avian uncoupling protein (avUCP) was significantly down-regulated by HS treatment. Conversely, in the *Gas* muscle of HS birds, SOD2 mRNA level was significantly increased while avUCP mRNA level was unchanged. Based on this evidence, it is suggested that the glycolytic muscle (e.g., *Gas* muscle) in broiler chickens is more susceptible to HS-induced oxidative disturbance, in which avUCP and SOD2 may be involved.

Introduction

Skeletal muscles are composed of two major muscle fibre types that are referred to as glycolytic (type II) and oxidative (type I) muscle fibres. A major difference between oxidative and glycolytic muscles is their mitochondrial content. Mitochondria function not only as a major energy plant but also as a reactive oxygen species (ROS) generator within most cells. Oxidative muscles are particularly susceptible to ROS attacks due to their high mitochondrial abundance, and therefore they have

higher activity of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Picard et al., 2012). This leads to specific mitochondrial oxidative phenotypes in histochemically and metabolically different muscle fibres.

Heat stress (HS) is an environmental factor that causes oxidative damage to skeletal muscle tissues in animals. Our previous studies have shown that HS induces oxidative damage (Kikusato et al., 2010), involving excess mitochondrial ROS generation due to a suppression of avian uncoupling protein (avUCP) expression (Kikusato and Toyomizu, 2013). These findings were observed in *Musculus pectoralis* (*Pec*) muscle, which consists entirely of glycolytic (type IIB) muscle fibres. Glycolytic muscle fibre has a lower mitochondrial content than oxidative muscle (Hakamata et al., 2018). There have been no comparative studies on oxidative disturbance and resistance in skeletal muscles with different muscle fibre composition of HS-exposed birds.

The present study evaluates the effect of HS on oxidative disturbance in two different types of muscles, *Pec* muscle and *gastrocnemius* (*Gas*) muscle that consists of 65% type IIB muscle fibres (Williams and Dhoot, 1992). In addition, this study investigates the possible involvement of mitochondrial ROS generation and antioxidative enzymes in HS-induced oxidative damage.

Material and methods

Animals and experimental design

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

Twelve 0-day-old male broiler chickens (Ross strain, *Gallus gallus domesticus*) were obtained from a commercial hatchery (Matsumoto Poultry Farms and Hatcheries Co., Ltd., Zao, Miyagi, Japan). Animals were housed in electrically-heated batteries under continuous light for 14 days and provided *ad libitum* access to water and a standard diet for meat-type chickens (crude protein 21%; metabolizable energy 3100 kcal/kg), with each ingredient meeting National Research Council (NRC, 2014) recommendation levels. Thereafter, chickens were randomly divided into two groups and moved to individual wire cages. At 25 day of age, the control group was maintained at thermoneutral conditions (TN) (24 °C) while the other group was exposed to HS conditions (34 °C) for 12 h. Both conditions were kept at 55–65% relative humidity. After the HS treatment, rectal and muscle temperature were measured by using digital thermometer equipped with a needle type probe. The birds were euthanized by decapitation, and the *Pec* and *Gas* muscles were excised and placed in an ice-cold medium comprising 100 mM KCl, 50 mM tris(hydroxymethyl) aminomethane (Tris)/HCl (pH 7.4) and 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) for mitochondrial isolation (see below). The muscles were frozen, powdered in liquid nitrogen, and stored at −80 °C until analysis.

Isolation of skeletal muscle mitochondria

Skeletal muscle mitochondria were isolated by homogenization, protein digestion and differential centrifugation at 4 °C, as described previously (Kikusato and Toyomizu, 2015b). The protein concentration of the isolated mitochondria was determined using the bicinchoninic acid assay, with bovine serum albumin as the standard. All mitochondria were freshly prepared on the day of the experiment. The respiratory control ratio (RCR) of each mitochondrial sample was measured by using 73 μM ADP to examine mitochondrial quality, as previously described (Kikusato and Toyomizu, 2015). The RCR is a ratio of the mitochondrial O_2 consumption rate in the presence of ADP to the rate in its absence. The RCR values for succinate-supported respiration were approximately 2.6–2.9 in all mitochondrial samples, meaning that the isolated mitochondria were of good quality for functional evaluation.

Determination of mitochondrial ROS production

Mitochondrial ROS generation rates were determined by measuring the hydrogen peroxide (H_2O_2) generation rate, which was fluorometrically measured by the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex™ Red, Invitrogen, Carlsbad, CA, USA) coupled to the enzymatic reduction by horseradish peroxidase, as described previously (Kikusato and Toyomizu, 2015).

Measurement of lipid peroxidation

Muscle lipid peroxidation was determined based on the level of thiobarbituric acid (TBA) reactive substances (TBARS). TBARS values were measured as previously described (Kikusato et al., 2016), and were expressed as nanomoles of malondialdehyde (MDA) per equivalent g of wet tissue.

Quantification of mRNA expression using Real-Time reverse transcription polymerase chain reaction (RT-PCR)

Isolation of tissue RNA and synthesis of cDNA were conducted as previously described (Kikusato et al., 2016). Real-time RT-PCR analysis was performed to quantify mRNA using a CFX Connect™ system (Bio-Rad Laboratories, Hercules, CA, USA). mRNA levels of the following ROS regulating proteins and the transcriptional co-factor were quantified: SOD1, SOD2, CAT, GPx4, avUCP and peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α). The expression values were normalized to that of 18S-ribosomal RNA (RN18S). Primer sequences of the genes were described in our other papers (Kikusato et al., 2015; 2016).

Statistical analysis

All data are presented as the mean ± standard error (SE) of 6 individual samples. Statistical differences between the TN and HS birds in each muscle were identified using a Student's *t*-test. Differences were considered significant for values of $P < 0.05$.

Results and discussion

In broiler chickens exposed to HS treatment TBARS values in *Pec* muscle were significantly increased, while in *Gas* muscle there was no increase noted (Figure 1A). These different responses are due to the different effects of HS on the muscles. The differences in muscle temperature could be possibly a factor. However, it was found that the increase in muscle temperature following HS treatment did not differ between the muscles (Δ2.4 ± 0.4 °C in *Pec* muscle; Δ1.7 ± 0.5 °C in *Gas* muscle). Also, we further compared the degrees of HS-induced oxidative damage (% increase in TBARS values of HS condition compared to TN condition) in *Pec*, *Gas* and *extensor digitorum longus* (type IIB: 87%) (Williams and Dhoot, 1992). HS-induced lipid peroxidation was shown to decrease as the percentage of glycolytic muscle fibre in the muscles decreased (data not shown), suggesting that oxidative status of muscles with a higher proportion of glycolytic muscle fibre might be more susceptible to HS.

Table 1. Gene expression levels of avian uncoupling protein (avUCP), superoxide dismutase (SOD) 2, SOD1, catalase (CAT), glutathione peroxidase (GPx) 4 and peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) genes in *M. pectoralis* (*Pec*) and *gastrocnemius* (*Gas*) muscles of thermoneutral (TN) and heat-stressed (HS) chickens

Genes	*Pec* muscle		*Gas* muscle	
	TN	HS	TN	HS
avUCP	1.00 ± 0.08	0.64 ± 0.05*	1.21 ± 0.08	1.18 ± 0.05
SOD2	1.00 ± 0.11	1.09 ± 0.15	1.53 ± 0.11	1.89 ± 0.10*
SOD1	1.00 ± 0.09	1.12 ± 0.08	1.27 ± 0.09	1.10 ± 0.08
CAT	1.00 ± 0.09	0.94 ± 0.10	0.96 ± 0.09	1.08 ± 0.10
GPx4	1.00 ± 0.13	1.11 ± 0.06	1.25 ± 0.13	1.29 ± 0.06
PGC-1α	1.00 ± 0.12	0.94 ± 0.07	1.41 ± 0.12	1.60 ± 0.07

Real-Time reverse transcription polymerase chain reaction (RT-PCR) was used to quantify the mRNA levels. The results were normalized to 18s-ribosomal protein (RN18S) transcript levels. Values are means ± error, n = 6, and data were expressed as relative values to *Pec* muscle of TN birds. * indicates the difference between TN and HS chickens for each muscle separately at $P < 0.05$.

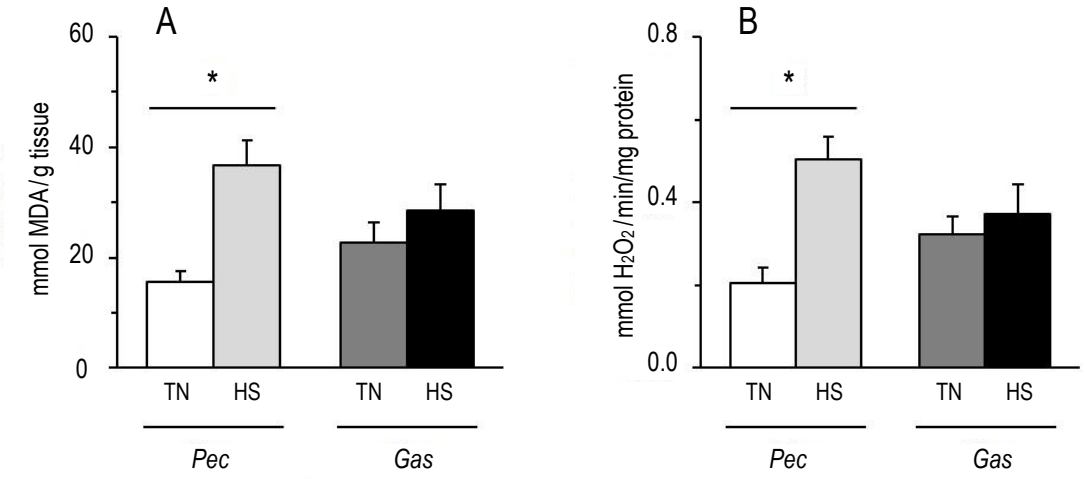

Figure 1. Lipid peroxidation (A) and mitochondrial H_2O_2 production (B) in *Pec* (*M. pectoralis*) and *Gas* (*gastrocnemius*) muscles in thermoneutral (TN) and heat-stressed (HS) broiler chickens

Lipid peroxidation was measured using thiobarbituric acid (TBA), and is expressed as nmol of malondialdehyde (MDA) per equivalent g wet tissue. H_2O_2 production rate in isolated mitochondria oxidizing succinate (4 mM) was fluorometrically determined using Amplex™ Red (Invitrogen, Carlsbad, CA, USA). Values are means ± standard error, n = 6. * indicates the difference between TN and HS chickens for each muscle separately at $P < 0.05$.

Apart from oxidative damage, in the present study it was shown that HS resulted in a significant increase in mitochondrial H_2O_2 production in *Pec* muscle, while in *Gas* muscle the increase was not observed (Figure 1B). Also, mechanism regulating mitochondrial ROS generation in these muscles under HS conditions was examined. It was found that down-regulation of avUCP, a mitochondrial inner membrane protein, evokes ROS generation through an increase in the inner membrane potential under hyperthermic conditions (Kikusato and Toyomizu, 2013).

It was shown that the avUCP mRNA level in *Pec* muscle was significantly decreased by the HS treatment, and did not change in *Gas* muscle (Table 1). Following the HS treatment, mRNA levels of SOD2, which is an antioxidative enzyme localized in the mitochondrial matrix, were unchanged in *Pec* muscle, while the levels of SOD2 in *Gas* muscle were significantly higher in HS birds than in TN birds. In addition, the study investigated the change in transcription rates of the cytosolic antioxidative enzymes: SOD1, CAT and GPx4; however, no significant differences in the mRNA levels were observed between TN and HS groups in both *Pec* and *Gas* muscles. These results suggest that maintained levels of avUCP and up-regulation of SOD2 may play an important role in the suppression of mitochondrial ROS generation and subsequent oxidative disturbance in skeletal muscle of HS-exposed broiler chickens.

Skeletal muscle fibre type transformation between fast and slow fibre types is caused by physiological stimuli in order to fulfil metabolic demands. The present study did not evaluate these changes in *Pec* and *Gas* muscles of HS-exposed birds; however, in a previous study it was reported that mild HS induces differentiation of mammalian myoblasts, shifting fibre type from glycolytic to oxidative through PGC-1α (Yamaguchi et al., 2010). PGC-1α, a transcriptional co-factor, plays a pivotal role in the muscle fibre type shift (Zhang et al., 2017); however, the present study found that PGC-1α mRNA levels were not altered by HS treatment in *Pec* and *Gas* muscles (Table 1). Therefore, it is unlikely that HS induced muscle fibre transformation in the present study. Given that PGC-1α induces the transcription of mitochondrial antioxidative factors (Valle et al., 2005), the mechanism maintaining avUCP mRNA level and up-regulating SOD2 in *Gas* muscle remains unclear. One might assume that other signalling molecules such as AMP-activated protein kinase (AMPK) and sirtuin-1, which act in coordination with PGC-1α in energy metabolism (Cantó and Auwerx, 2009), or the higher intrinsic levels of PGC-1α mRNA (*Gas* muscle vs *Pec*, $P < 0.05$), might be associated with the transcription of the mitochondrial antioxidative factors in *Gas* muscle of HS-exposed birds. Further investigation is required to clarify if specific muscles have a higher tolerance to oxidative disturbance caused by HS treatment.

The present study revealed that the oxidative status of *Gas* muscles did not respond to acute HS treatment. However, further investigations are needed to verify whether this finding is applicable to other animals. In a study on pigs it was reported that oxidative muscle is more susceptible to HS-induced changes in redox balance than glycolytic muscle during chronic HS (Rosado Montilla et al., 2014). Conversely, it was reported that HS-induced oxidative disturbance did not differ considerably between muscles with different fibre types: *Gas*, *soleus,* and *plantaris* muscles, in aged mice (Tamura et al., 2017). It is conceivable that the difference in HS intensity and duration between the studies might be associated with the discrepancies between these results, while one might assume that the muscle lipid content of the different animals used affected the degrees of HS-exposed oxidative damage.

Conclusions

It is suggested that skeletal muscles of broiler chickens that contain different amounts of glycolytic muscle fibre exhibit different responses to heat stress (HS)-induced oxidative disturbance, in which the mitochondrial antioxidative factors such as avian uncoupling protein (avUCP) and superoxide dismutase 2 (SOD2) may be involved.

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