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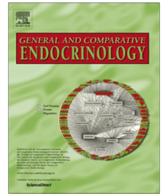

Time-course changes in muscle protein degradation in heat-stressed chickens: Possible involvement of corticosterone and mitochondrial reactive oxygen species generation in induction of the ubiquitin–proteasome system

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ABSTRACT

Heat stress (HS) induces muscle protein degradation as well as production of mitochondrial reactive oxygen species (ROS). In the present study, to improve our understanding of how protein degradation is induced by HS treatment in birds, a time course analysis of changes in the circulating levels of glucocorticoid and N^{τ}-methylhistidine, muscle proteolysis-related gene expression, and mitochondrial ROS generation, was conducted. At 25 days of age, chickens were exposed to HS conditions (33 °C) for 0, 0.5, 1 or 3 days. While no alteration in plasma N^{τ}-methylhistidine concentration relative to that of the control group was observed in the 0.5 day HS group, the concentration was significantly higher in the 3-d HS treatment group. Plasma corticosterone concentrations increased in response to 0.5-d HS treatment, but subsequently returned to near-normal values. HS treatment for 0.5 days did not change the levels of μ-calpain, cathepsin B, or proteasome C2 subunit mRNA, but increased the levels of mRNA encoding atrogin-1 ($P < 0.05$) and its transcription factor, forkhead box O3 ($P = 0.09$). Under these hyperthermic conditions, mitochondrial superoxide production was significantly increased than that of thermoneutral control. Here, we show that HS-induced muscle protein degradation may be due to the activation of ubiquitination by atrogin-1, and that this process may involve mitochondrial ROS production as well as corticosterone secretion.

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1. Introduction

Hyperthermia, also referred to as heat stress (HS), induces muscle protein degradation. It is widely accepted that increased levels of circulating corticosterone, which is a primary glucocorticoid secreted in avian species, may be associated with protein degradation in HS-exposed birds (Yunianto et al., 1997). Several investigations have reported that glucocorticoid treatment induces the expression of muscle atrogenes such as atrogin-1 and MuRF1 (Menconi et al., 2008; Nakashima et al., 2015), both of which act as ubiquitin ligases in the ubiquitin–proteasome protein degradation system (UPS), and that the level of muscle atrogin-1 mRNA is increased in response to HS treatment (Boussaid-Om Ezzine et al., 2010). In addition, the UPS is activated in sepsis (Wray et al., 2003) that drives hyperthermia, and glucocorticoid secretion is thought to be implicated in this induction of the UPS (Tiao et al., 1996, 1997). Despite these lines of evidence, there has been no systematic investigation of the hormonal and proteolytic alterations which result from HS treatment.

In contrast to the possible role of corticosterone secretion in muscle proteolysis, recent research has proposed that mitochondria-derived reactive oxygen species (ROS) play an important role in intracellular signal transduction (Finkel, 2011), through which they also seem to contribute to protein catabolism (Li et al., 2003; Gilliam et al., 2012; Rahman et al., 2014). Our recent investigation demonstrated that mitochondrial superoxide production induces the production of atrogin-1 mRNA, resulting in UPS-dependent protein degradation in HS-treated cultured muscle cells (Furukawa et al., 2015). These findings allow us to hypothesize that mitochondrial ROS induced by the HS treatment may induce the protein degradation. In addition, given that

Abbreviations: HS, heat stress; ROS, reactive oxygen species; UPS, ubiquitin–proteasome system; FoxO, forkhead box O; GP, glycerol 3-phosphate; 18S, 18S ribosomal RNA; JNK, c-Jun-N-terminal kinase.

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glucocorticoid administration causes oxidative disturbance, it is possible that glucocorticoid secretion evokes the mitochondrial superoxide production by which muscle protein degradation is induced via activation of the UPS (Lin et al., 2004a,b; Oshima et al., 2004).

In order to clarify the mechanism whereby muscle protein degradation is induced by HS treatment, we monitored changes in the circulating levels of glucocorticoids and plasma N^{τ}-methylhistidine concentrations which are used to evaluate protein degradation (Ijiri et al., 2014), and the levels of mRNA encoding atrogin-1 and other proteolysis-related proteins. Furthermore, we evaluated changes in muscle mitochondrial superoxide production in response to HS treatment. We show that HS treatment induced plasma corticosterone secretion and muscle mitochondrial superoxide production as well as atrogin-1 gene expression. Increases in these parameters occurred at the same time point, suggesting that HS-induced muscle proteolysis could be subject to both hormonal and redox regulation.

2. Materials and methods

2.1. 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.

2.2. Animals and experimental design

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). They 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 content, 3100 kcal/kg). Thereafter, chickens were randomly divided into two groups and moved to individual wire cages. At 25 d of age, the control group was maintained at thermoneutral conditions (24 °C) while the other group was exposed to HS conditions (33 °C). This involved increasing the temperature by 1 °C per hour until it reached 33 °C. After 0, 0.5, 1 or 3 days of the experiment, the birds were killed by decapitation. The *pectoralis superficial* muscle and plasma were excised/harvested and immediately frozen, powdered in liquid nitrogen, and stored at −80 °C until analyzed.

2.3. Determination of plasma N^{τ}-methylhistidine content

The rate of skeletal muscle protein degradation was estimated by measuring the concentration of N^{τ}-methylhistidine in plasma over time as described previously (Hayashi et al., 1987; Kamizono et al., 2010, 2015). Plasma was mixed with 200 g/L sulphosalicylic acid and centrifuged at 9600*g* for 5 min. The supernatant was recovered and evaporated under reduced pressure. The residue was dissolved in 0.2 M pyridine and applied to a cation-exchange column (7 × 60 mm, Dowex 50 W-X8, 200–400 mesh, pyridine form). After most of the acidic and neutral amino acids were washed out with 0.2 M pyridine, N^{τ}-methylhistidine was eluted with 1 M pyridine and collected. The solvent was evaporated and the residue dissolved in the mobile phase (15 mM sodium 1-octanesulfonate in 20 mM KH_2PO_4). An aliquot was injected into an HPLC (LC-6A, Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-80A column (4.6 × 250 mm, 5 µm, GL Sciences, Tokyo, Japan). The column was inserted into a column oven at 50 °C. A fluorescence detector (RF-535, Shimadzu, Kyoto, Japan), using an excitation wavelength of 365 nm and emission wavelength of 460 nm, was used to monitor the fluorescence derived from the reaction with *o*-phthalaldehyde.

2.4. Determination of plasma corticosterone concentration

Plasma corticosterone concentration was analyzed by using a commercially-available ELISA kit (Enzo Life Sciences, ADI-900-097). The experiment was performed according to the manufacturer's instructions.

2.5. Quantification of mRNA expression using real-time polymerase chain reaction (RT-PCR)

Isolation of tissue RNA and synthesis of cDNA were conducted as previously described (Kikusato et al., 2015). 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 proteolysis-related genes were quantified: atrogin-1; proteasome C2 subunit, a major component of the 20S proteasome in the UPS; and µ-calpain and cathepsin B, major enzymes of the calpain and lysosome systems. The mRNA levels of forkhead box O (FoxO)1 and FoxO3, which are transcription factors of atrogin-1, were also measured. Primer sets used to amplify each gene are listed in Table 1. To correct for differences in the amount of template cDNA used, the results are presented as ratios of the target mRNA to 18S ribosomal RNA (18S) levels. All data are shown as fold changes relative to the values measured at day 0 of treatment.

2.6. Determination of mitochondrial superoxide production

Skeletal muscle (*pectoralis superficial* muscle) mitochondria were isolated from 0.5 day HS-treated birds by homogenization, protein digestion and differential centrifugation at 4 °C, as described previously (Kikusato and Toyomizu, 2015). The mitochondrial protein concentration was determined using the bicinchoninic acid assay with bovine serum albumin (BSA) as the standard. All mitochondria were freshly prepared on the day of the experiment. Mitochondrial superoxide generation rates were determined as the hydrogen peroxide (H_2O_2) generation rate, which was fluorometrically measured by the oxidation of 10-acet yl-3,7-dihydroxyphenoxazine (Amplex® Red, Invitrogen) coupled to enzymatic reduction by horseradish peroxidase (HRP), as described previously (Kikusato and Toyomizu, 2015). Mitochondria were incubated in assay medium (80 mM KCl, 50 mM Hepes (pH 7.2), 1 mM EGTA, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.3% (*w/v*) defatted

Table 1
Primer sequences.

Gene		Primer sequence (5′–3′)	Gene No.
Atrogin-1	Sense	CCA ACA ACC CAG AGA CCT GT	NM_001030956.1
	Antisense	GGA GCT TCA CAC GAA CAT GA	
Proteasome C2 subunit	Sense	AAC ACA CGC TGT TCT GGT TG	AF027978
	Antisense	CTG CGT TGG TAT CTG GGT TT	
µ-Calpain	Sense	GGG CTA CAA ACT GAC CCA AA	NM_001044672.1
	Antisense	TAG GCA AAC ATG GTG AGC TG	
Cathepsin B	Sense	CAA GCT CAA CAC CAC TGG AA	NM_205371
	Antisense	TCA AAG GTA TCC GGC AAA TC	
FoxO1	Sense	ATT GAG CTG GCA AAA GTG CT	NM_204328.1
	Antisense	GCT GTC AAC ATG CCT CTC AA	
FoxO3	Sense	CGT TGT CAG TCT GAA TGT GGG G	XM_001234495.3
	Antisense	GAC AGC AGA TTT GGC AAA GGG	
18S	Sense	TAG ATA ACC TCG AGC CGA TCG	AF173612.1
	Antisense	GAC TTG CCC TCC AAT GGA TCC	

Abbreviations: FoxO, forkhead box O; 18S, 18S ribosomal RNA.

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