Acute Heat Stress Induces Oxidative Stress and Decreases Adaptation in Young White Leghorn Cockerels by Downregulation of Avian Uncoupling Protein

A. Mujahid, Y. Akiba, and M. Toyomizu

Science of Biological Function, Life Science, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 981-8555, Japan

ABSTRACT Reactive oxygen species-induced damage of cells and molecules is one of the mechanisms responsible for the decline in an animal’s performance due to heat stress. Mitochondria are the main producers of cellular superoxide, a process that is sensitive to proton motive force, and this superoxide production can be decreased by mild uncoupling. We studied the effects of heat stress on the production of mitochondrial superoxide as well as heat stress effects on the expression of avian uncoupling protein (avUCP) and avian A nucleotide translocator (avANT) in skeletal muscles of chicks and young cockerels. Male White Leghorn (Julia) chicks at 16 d and cockerels at 87 d of age were exposed to acute heat stress, 34°C for 18 h, or kept at moderate ambient temperature (25 and 21°C, respectively). There was no difference in mitochondrial superoxide production between heat-exposed and control chicks, whereas significant differences were observed in the case of young cockerels. Greater substrate-independent superoxide production was found in muscle mitochondria from heat-stressed young cockerels. In chicks, neither avUCP nor avANT transcript expression was changed by heat exposure, whereas in young cockerels avUCP transcript was decreased, but avANT transcript level was not changed. Thus, in heat-stressed young cockerels, increased mitochondrial superoxide production was accompanied by downregulation of avUCP. Taken together, these results suggest that exposure of young cockerels to heat stress stimulates mitochondrial superoxide production, possibly via downregulation of avUCP. Chicks with persistent avUCP expression, on the other hand, are relatively better adapted to high temperature. It can be assumed that appropriate expression of avUCP may alleviate overproduction of mitochondrial superoxide and could help birds adapt to oxidative stress resulting from acute heat stress.

Key words: uncoupling protein, heat stress, oxidative stress, adaptation, chicken

INTRODUCTION Heat stress causes serious physiological dysfunction that may result in a decline in animal performance. Hyperthermia has been proposed to be responsible for stimulating reactive oxygen species (ROS) production because of similarities in the expression patterns of genes (including heat shock, oxidative stress proteins, or both) observed following heat stress compared with that following exposure to oxidative stress (Schiaffonati et al., 1990; Salo et al., 1991). In chickens, oxidative stress was observed on exposure to acute heat stress (Mujahid et al., 2005b; Lin et al., 2006). We provided direct evidence of mitochondrial ROS generation in the skeletal muscle of heat-stressed chickens by using both electron spin resonance spectroscopy, with 5,5-dimethyl-1-pyrroline N-oxide as a spin trap agent and lucigenin-derived chemiluminescence (LDCL; Mujahid et al., 2005b). More recently, we found that this increase in mitochondrial ROS production during the heat stress period was substrate-independent (Mujahid et al., 2006). In mitochondria, superoxide anion and other ROS are thought to be produced as inevitable byproducts of normal aerobic metabolism. The primary source of ROS is leakage of electrons from the respiratory chain during the reduction of molecular oxygen to water, to generate superoxide anion (Boveris et al., 1972). Superoxide is a reactive molecule, but it can be converted to hydrogen peroxide by superoxide dismutase and then to oxygen and water by catalase or glutathione peroxidase. Although ROS may play an important role in cellular functions such as cell signaling, it is obvious that high levels of ROS also cause cellular damage (Beckman and Ames, 1998), at least at the mitochondrial level (Raha and Robinson, 2000).

Superoxide production is sensitive to proton motive force and can be decreased by mild uncoupling (Brand et al., 2004). Mammalian uncoupling proteins (UCP) belong to a family of transporter proteins present in the
OXIDATIVE STRESS AND UNCOUPLING PROTEIN IN CHICKENS

Figure 1. Possible role of uncoupling protein (UCP) and A nucleotide translocator (ANT) in control of superoxide production in mitochondria. A mitochondrial proton gradient generated by complexes of respiratory chain across inner mitochondrial membrane is used by 2 mechanisms. First, it is used by adenosine triphosphate (ATP) synthase to phosphorylate adenosine diphosphate (ADP) and produce ATP (A). This oxidative phosphorylation is the main consumer of the proton gradient. Second, another mechanism consuming the proton gradient is proton leak (B). When the proton gradient is dissociated by UCP or ANT, the reentry of protons into the matrix is not coupled to ATP synthesis. In this way, UCP and ANT cause mitochondrial proton leak, resulting in reduction of the proton gradient. During the process of oxidative phosphorylation some electrons leak out from the electron transport chain (ETC) and combine with molecular oxygen to generate superoxide (C). This superoxide production is very sensitive to the proton gradient. Mild uncoupling that reduces the proton gradient can result in decreased superoxide production at the expense of a small loss of energy. FADH2 = reduced flavin A dinucleotide; FAD+ = flavin A dinucleotide.

mitochondrial inner membrane that, by dissipating the mitochondrial proton gradient, uncouple respiration from adenosine triphosphate synthesis (Palmieri, 1994). Himms-Hagen (1985) found that UCP1 is present mainly in brown adipose tissue, which is the major site of regulatory thermogenesis in small rodents. Five additional uncoupling protein homologs, UCP2 to UCP4, brain mitochondrial carrier protein type 1, and kidney mitochondrial carrier protein 1 have been identified to date. Fleury et al. (1997) found that UCP2 is expressed ubiquitously, whereas UCP3 gene expression is seen in skeletal muscle, adipose tissue, and heart (Boss et al., 1997; Acin et al., 1999). Brain mitochondrial carrier protein type 1, kidney mitochondrial carrier protein 1, and UCP4, all of which have recently been identified (Sanchis et al., 1998; Mao et al., 1999; Haguenauer et al., 2005), are expressed primarily in the brain, other neural tissues, and within the kidney cortex.

Although bird species have no distinct stores of brown adipose tissue or a related type of thermogenic tissue (Johnston 1971; Saarela et al., 1991), a new protein named avian uncoupling protein (avUCP), which shares 71 to 73% amino acid homology with both UCP2 and UCP3, was identified in chicken skeletal muscles (Raimbault et al., 2001; Toyomizu et al., 2002). Little is known of the precise physiological roles of both mammalian UCP2 and UCP3 and avUCP. It is thought that UCP could play a role in the mediation of thermogenesis, in the utilization of lipids as fuel substrates, in the control of insulin secretion, and in controlling the production of ROS and protecting against the deleterious effect of ROS (Adams, 2000; Collin et al., 2003; Criscuolo et al., 2005). As shown in Figure 1, it should be noted that mild mitochondrial uncoupling via the action of A nucleotide translocator (ANT), as well as UCP, in skeletal muscle may play a role in alleviating the generation of harmful ROS for which an increased mitochondrial flux may occur (Sklachev, 1998; Echtay et al., 2003).

On exposure to acute heat stress, we found that White Leghorn (WLH) chickens responded differently as compared with broiler chickens of the same age (Mujahid et al., 2005b). More recently, we provided evidence that synthesis of avUCP protein was downregulated in heat-stressed broilers and suggested that acute heat stress stimulates mitochondrial superoxide production in muscle, possibly via downregulation of avUCP (Mujahid et al., 2006). The role of avUCP in WLH chickens in controlling superoxide production under heat stress is not well known, although the role of avUCP during cold stress (Mujahid et al., 2005a; Ueda et al., 2005) and fasting (Abe
et al., 2006; Toyomizu et al., 2006) in WLH chickens has been extensively studied. The present study was therefore carried out to clarify the effects of acute heat stress on mitochondrial superoxide production and expression of avUCP and avian A nucleotide translocator (avANT) in skeletal muscle of chicks and young cockerels of the WLH strain.

MATERIALS AND METHODS

Birds and Experimental Design

Laying-type male chicks (Julia) were obtained from a commercial hatchery (Economic Federation of Agricultural Cooperatives, Iwate, Japan) at 1 d of age. The chicks were housed in electrically-heated batteries under standard husbandry conditions with continuous light and provided with ad libitum access to water and commercial diet according to the manufacturer’s recommendations. Sixteen-day-old chicks (n = 4 to 8) and 87-d-old young cockerels (n = 6) were used in the first and second series of experiments, respectively, and the birds were subjected to acute heat stress (34°C for 18 h). The control birds were kept at moderate ambient temperatures (25 and 21°C, respectively). Birds were killed by decapitation, and pectoralis superficialis muscles were rapidly excised. This method of killing was used in preference to overdose by general anesthetics, which are known to uncouple oxidative phosphorylation (Rottenberg, 1983). For isolation of mitochondria, muscles were placed in ice-cold isolation buffer A (see below). To study the expression of genes, muscles were frozen, powdered in liquid N, and stored at -80°C until required for extraction of total RNA. All experiments were performed in accordance with institutional guidelines concerning animal use.

Isolation of Mitochondria

Muscle subsarcolemmal (SS) mitochondria were isolated from pectoralis superficialis as previously described (Toyomizu et al., 2002). Muscles were trimmed of fat and connective tissue, blotted dry, weighed, and then minced with scissors. The minced tissue was suspended in ice-cold buffer A [containing 100 mM sucrose, 50 mM Tris base, 5 mM MgCl2, 5 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N′,N′,N″-tetra-acetic acid (EGTA), 100 mM KCl, pH 7.4] and homogenized with a Potter-Elvehjem homogenizer (5 strokes, Iwaki Glass Co. Ltd., Tokyo, Japan). The homogenate was then centrifuged at 800 × g for 10 min. The supernatant was centrifuged at 1,000 × g for 10 min and then 8,700 × g for 10 min. The resulting pellet, containing SS mitochondria, was suspended in buffer A and recentrifuged at 8,700 × g for 10 min. The resulting pellet was resuspended in buffer B (containing 250 mM sucrose, 20 mM Tris base, 1 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N′,N′,N″-tetra-acetic acid, pH 7.4) and then washed by centrifugation at 8,700 × g for 10 min. The final SS mitochondrial pellet was suspended in a minimal volume of buffer B and kept on ice. All procedures were carried out at 4°C. Mitochondrial protein concentration was measured by the Lowry method.

Mitochondrial Superoxide Production

The LDCL method, using a Berthold luminometer (Mujahid et al., 2005b), was performed to measure superoxide anions produced by the mitochondria isolated from the pectoralis superficialis of control and heat-stressed birds. The reaction conditions were as follows: 70 mM sucrose, 220 mM mannitol, 2 mM N-2-hydroxyethylpiperazine-N″-2-ethanesulfonic acid, 2.5 mM potassium phosphate, 0.5 mM EDTA, pH 7.4, 20 μM lucigenin, and 0.5 mg/mL of mitochondria. After recording background LDCL for 4 min, the assay was initiated by the addition of 5 mM malate and 10 mM glutamate for complexes I, III, and IV of the electron transport chain, or 5 mM succinate + 5 μM rotenone for complexes II, III, and IV. Lucigenin-derived chemiluminescence was recorded at 2-s intervals for 5 min, and the data were expressed as the area under the curve calculated by integration.

Quantitation of mRNA Using Reverse Transcription PCR

Standard molecular biological techniques were used, essentially, as described by Sambrook et al. (1989). Tissues were homogenized in Trizol Reagent (Invitrogen, San Diego, CA) and total RNA isolated according to the manufacturer’s protocol. To study changes in expression of mRNA of avUCP and avANT, real-time reverse transcription PCR analyses were performed using the iCycler Real-Time Detection System (Bio-Rad Laboratories Inc., Hercules, CA). Five micrograms of total RNA, prepared using Trizol Reagent (Invitrogen), was reverse-transcribed using a mixture of oligo(dT)12-18 and random primers and Moloney murine leukemia virus reverse transcription (Invitrogen). One microliter of each reverse transcription reaction product then served as a template in a 50-μL PCR reaction containing 2 mM MgCl2, 0.5 μM each primer, and 0.5× SYBR green master mix (BioWhittaker Molecular Applications, Rockland, ME). The SYBR green fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. At the end of each run, melting curve profiles were recorded. Oligonucleotide sequences of sense and antisense primers and annealing temperatures were determined based on our earlier paper (Mujahid et al., 2006). The specificity of the amplification product was further verified by electrophoresis on a 0.8% agarose gel and by DNA sequencing. Results are presented as the ratio of mRNA to 18S ribosomal RNA to correct for differences in the amounts of template cDNA used.

Statistical Analysis

Data were analyzed using the Statistical Analysis System (SAS Inst. Inc., Cary, NC). Differences between heat-
Figure 2. Weight gain and feed consumption of chicks and young cockerels exposed to heat (34°C for 18 h) or kept at moderate ambient temperature. Values represent the mean ± SE for 6 to 8 birds in each group. *P < 0.05 for heat-stressed group vs. control group.

RESULTS AND DISCUSSION

Heat Stress Decreases BW Gain and Feed Consumption

The BW gain of chicks decreased on exposure to heat (Figure 2) and was less than that of control chicks. The percentage gain in BW for heat-exposed chicks was 2.2% vs. the control chicks with a 4.5% gain (P < 0.05). In contrast, the BW gain of young cockerels was severely suppressed on exposure to heat stress. Young cockerels lost 3.4% of their BW on exposure to heat stress for 18 h, compared with control birds, which had a 1.8% weight gain. On exposure to heat, consumption of feed decreased (P < 0.05) in both chicks and young cockerels (Figure 2). Feed consumption was more severely suppressed in heat-stressed young cockerels (87.4%) than heat-exposed chicks (34.1%); however, both showed a reduction in feed consumption when compared with their respective controls. Although weight gain and feed consumption were decreased in both chicks and young cockerels as a result of heat stress, chicks performed comparatively better than young cockerels.

Heat Stress Induces Substrate-Independent Superoxide Production in Mitochondria

Oxidizing glutamate and malate, skeletal muscle (pectoralis superficialis) mitochondria generated a higher level of superoxide in young cockerels exposed to heat stress than control cockerels. However, no difference was observed between heat-exposed chicks and control chicks (Figure 3). To examine whether the increased superoxide production in the skeletal muscle of the heat-stressed young cockerels was substrate-dependent, we measured the superoxide production of skeletal muscle mitochondria using different substrates. Glutamate-requiring complexes I, III, and IV of the electron transport chain and succinate-requiring complexes II, III, and IV were used as substrates. Significant increases in superoxide production by mitochondria isolated from skeletal muscle were observed in heat-stressed young cockerels compared with that of control birds, regardless of the substrates used (NAD O$_2$ or flavin A dinucleotide-linked substrates, Figure 3, panel B).

Reactive oxygen species include free radical species (i.e., unpaired, electron-containing species), such as pri-
Figure 3. Mitochondrial superoxide radical production. Lucigenin-derived chemiluminescence (LDCL) analysis was carried out using subsarcolemmal mitochondria isolated from pectoralis superficialis muscle of control and heat-exposed (34°C for 18 h) chicks and young cockerels. Mitochondrial superoxide radical production in chicks using NAD-linked substrates (panel A). Mitochondrial superoxide radical production in young cockerels using NAD- or flavin A dinucleotide-linked substrates (panel B). The integrated areas under the curves are expressed as a ratio of mitochondrial protein. Values represent the mean ± SE of data from 4 birds each. *P < 0.05 for heat-stressed group vs. control group.

Primary superoxide (O$_2^-$) and its conjugated acid-hydroperoxyl radical (HO$_2$). Also included are the hydroxyl (·OH), carbonate (CO$_3^-$), peroxyl (RO$_2^-$), and alkoxyl (RO·) radicals. In addition, some nonradical species are ascribed to ROS, namely H$_2$O$_2$, HOCl, alkyl hydroperoxides (ROOH), reactive aldehydes, singlet oxygen, and other compounds (Halliwell and Gutteridge, 2002). In the process of ROS production, some electrons passing through the mitochondrial electron transport chain (ETC) leak out and react with molecular oxygen to form superoxide, which is quickly dismutated by the mitochondrial superoxide dismutase to H$_2$O$_2$ (Boveris et al., 1972). Thus, because O$_2^-$ is the first product synthesized in the process of ROS production and is then converted to hydroxyl radicals and lipid peroxides, it would clearly serve as an indicator of the response of muscle mitochondrial ROS production under heat stress conditions. On this basis, the LDCL method was applied to assess O$_2^-$ production in isolated mitochondria. Glutamate and malate-requiring complexes I, III, and IV of the ETC and succinate plus rotenone-requiring complexes II, III, and IV were used as substrates, because a significant proportion of the oxygen molecules are converted to O$_2^-$ by complexes I and III via a nonenzymatic process (Castella et al., 2001).

We reported a significant increase in superoxide anion production in the mitochondria isolated from the skeletal muscle of the heat-stressed broilers when either glutamate-requiring complexes I, III, and IV of the ETC and succinate plus rotenone-requiring complexes II, III, and IV were used as substrates, because a significant proportion of the oxygen molecules are converted to O$_2^-$ by complexes I and III via a nonenzymatic process (Castella et al., 2001).

Heat Stress Downregulates avUCP Transcripts in Young Cockerels

By using real-time reverse transcription PCR, avUCP (Figure 4, upper panels) and avANT (Figure 4, lower panels) transcripts were analyzed in pectoralis muscle of control and heat-exposed chicks and young cockerels. In chicks, gene transcript expression levels for avUCP and avANT were similar between control and heat-exposed chicks. In young cockerels, gene transcript expression for avUCP was significantly decreased to 34% of control levels after exposure to heat stress. In contrast, avANT transcript expression was not changed on exposure to heat stress (Figure 4).

It is widely accepted that mitochondria play a key role in the development of oxidative stress. The major endogenous sources of ROS are localized to mitochondria and can be related to the respiratory chain, substrate dehydrogenases in the matrix, monoamine oxidase, and cytochrome P450. On the other hand, significant antioxidant capacity is inherent in mitochondria (ubiquinol, Mn superoxide dismutase, glutathione- and thioredoxin-dependent systems). As a result, mitochondria could either am-
plify or suppress the general oxidative stress response provoked by exogenous stimuli. Skulachev (1996) showed for the first time the concept of mild uncoupling as a line of antioxidant defense and that minor UCP might mediate this uncoupling (Skulachev 1998) and thus decrease mitochondrial production of ROS (Papa and Skulachev, 1997; Rolfe and Brand, 1997). On this basis, UCP and ANT may be key regulators of mitochondrial ROS production (Figure 1). In terms of UCP, Vidal-Puig et al. (2000) and Brand et al. (2002) showed increased production of ROS and significantly higher levels of oxidative damage in UCP3 knockout mitochondria, respectively. Recently, we reported that in heat-stressed broilers, avUCP gene expression and its protein levels in mitochondria were downregulated, resulting in more ROS production by skeletal muscle mitochondria when compared with that of control birds (Mujahid et al., 2006). These results are supported by the current study; in WLH chicks showing no change in expression of avUCP gene on exposure to heat stress, their mitochondrial superoxide production was similar. However, in young cockerels, exposure to heat stress caused downregulation of avUCP transcript expression, resulting in significantly higher production of mitochondrial superoxide.

To determine whether skeletal muscle avANT is another key regulator of ROS flux under heat stress conditions, we also studied the possible contribution of avANT in suppressing ROS production via an uncoupling action in heat-exposed birds. It was shown that knocking out 1 of 2 ANT isoenzymes (muscle-specific ANT1) resulted in a strong increase in ROS production by muscle mitochondria of mice (Esposito et al., 1999). In the present study, we found that exposure of WLH chicks and young cockerels to heat did not significantly affect avANT transcript expression in the skeletal muscles (Figure 4, lower panels). The percentage increase in superoxide production in the presence of carboxyatractylate, a specific inhibitor of ANT, was similar for the skeletal muscle mitochondria of both control and heat-stressed broilers (Mujahid et al., 2005b). Recently, we also reported that avANT mRNA expression was similar between control and heat-stressed broiler chickens (Mujahid et al., 2006). These results suggest that skeletal muscle avANT may not be intensively involved in the regulation of superoxide production in the skeletal muscle of heat-stressed chickens, although further studies are required to elucidate the role of avANT under heat stress condition.

In conclusion, there was no change in mitochondrial superoxide production between heat-exposed and control chicks, whereas significant differences were observed in the case of young cockerels. Substrate-independent overproduction of superoxide occurred in mitochondria of heat-stressed young cockerels. In chicks, neither avUCP nor avANT transcript expression was changed by heat

Figure 4. Expression of mRNA for avian uncoupling protein (avUCP) and avian A nucleotide translocator (avANT) in the pectoralis superficialis muscle of control and heat-exposed (34°C for 18 h) chicks and young cockerels. Values are expressed as the means ± SE of 6 to 8 birds in each group. Real-time reverse transcription PCR was performed using primers designed based on the sequences registered in GenBank, as described in the Materials and Methods section. Results were normalized to 18S ribosomal RNA (rRNA) transcript levels. *P < 0.05 for heat-stressed group vs. control group. AU = arbitrary unit.
stress exposure, whereas in young cockerels, avUCP transcript level was decreased, but avANT transcript expression was not changed.

Taken together, these results suggest that exposure of young cockerels to heat stress stimulates mitochondrial superoxide production, possibly via downregulation of avUCP. Chicks with persistent avUCP expression, on the other hand, are relatively better adapted to high temperature. It can be assumed that appropriate expression of avUCP may alleviate overproduction of mitochondrial superoxide and could help adaptation to oxidative stress when birds are exposed to acute heat stress.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid (no. 18380157) for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES


