Sequential changes in superoxide production, anion carriers and substrate oxidation in skeletal muscle mitochondria of heat-stressed chickens

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Abstract We have shown that heat-stressed birds exhibit increased superoxide production in skeletal muscle mitochondria. To determine the precise mechanism for this effect, here we studied not only progressive, but also sequential changes in superoxide production, anion carriers and substrate oxidation in mitochondria of heat-stressed chickens. Exposure to acute heat stress (34 °C for 6, 12 and 18 h) stimulated pectoralis muscle mitochondrial superoxide production. Heat stress-induced down-regulations of avUCP gene transcripts and mitochondrial avUCP protein content were time-dependent: avUCP gene transcript was decreased after 6 h, while avUCP protein content was only downregulated after 12 h of heat stress. Avian adenine nucleotide translocator (avANT) gene transcripts were not changed on exposure to heat stress, suggesting that avANT may not be involved in the regulation of superoxide production in the muscle mitochondria of heat-stressed chickens. During the initial stage of acute heat stress β-oxidation enzymes gene transcripts and activity were upregulated, with elevated plasma non-esterified fatty acid levels and increased expression of mitochondrial fatty acid transport genes. This sudden surge in mitochondrial substrate oxidation resulted in higher superoxide production: the avUCP expression at 6 h after heat stress might have not been large enough to alleviate the overproduction of reactive oxygen species (ROS) even though a small amount of endogenous FFA, a potential uncoupler, might have been present in the mitochondria. Thereafter, avUCP content was downregulated while substrate oxidation returned to control levels. This downregulation of avUCP may have caused increased mitochondrial superoxide production, keeping the superoxide production rate high in the later stages of heat stress. These results suggest that overproduction of mitochondrial ROS in chicken skeletal muscle under the heat stress might result from enhanced substrate oxidation and downregulation of avUCP in a time-dependent manner.

1. Introduction

Oxidative stress, resulting from disturbed free-radical and cellular redox balance, is associated with ageing and several human diseases [1]: reactive oxygen species (ROS) can attack and damage surrounding biomolecules, for which accumulation of this damage may underlie some neurodegenerative diseases and is linked to aging processes [2]. Mitochondria are the primary source of ROS [3], with superoxide, which is produced at complexes I and III of the electron transport chain (ETC) [4], quantitatively the most important species. A mechanistic understanding of the overproduction of mitochondrial superoxide due to any physiologically abnormal condition is of great importance.

Heat stress, which is of concern for animal production as well as human life, especially in hotter regions of the world, is an environmental factor that could be responsible for stimulating ROS production. There are similarities in gene expression patterns observed following heat stress compared with that following exposure to oxidative stress [5,6]. Indeed we have already provided direct evidence of mitochondrial superoxide generation using both electron spin resonance (ESR) spectroscopy, with 5,5-dimethyl-1-pyrroline N-oxide as a spin trap agent, and lucigenin-derived chemiluminescence (LDCL) in skeletal muscle of acute heat-stressed chickens [7]. It was also shown that the acute heat treatments exhibited oxidative damage to mitochondrial proteins and lipids in chicken skeletal muscle [8]. However, the mechanism underlying acute heat stress-induced in vivo overproduction of mitochondrial superoxide in chicken skeletal muscle is not still clear.

Superoxide production by the ETC is favored by high cellular oxygen content and/or a highly reduced state of the ETC. Therefore, mechanisms that reduce membrane potential could lower superoxide production. A negative regulation of the mitochondrial ROS production by UCPs was first suggested in 1997 [9]; Skulachev in 1998 [10] also suggested that superoxide production may be reduced by factors that decrease mitochondrial membrane potential. Thus, as superoxide production can be

Keywords: Adenine nucleotide translocator; Carnitine palmitoyl transferase; Fatty acid oxidation enzyme; Heat stress; Reactive oxygen species; Uncoupling protein

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Abbreviations: 3HADH, 3-hydroxyacyl CoA dehydrogenase; ANT, adenine nucleotide translocator; CPT-I, carnitine-palmitoyl-transferase-I; CPT-II, carnitine-palmitoyl-transferase-II; CS, citrate synthase; FA, fatty acid; HNE, 4-hydroxynonenal; LCAD, long-chain acyl CoA dehydrogenase; LDCL, lucigenin-derived chemiluminescence; MACP, mitochondrial anion carrier protein; NEFA, non-esterified fatty acid; ROS, reactive oxygen species; SS, subsarcolemmal; UCP, uncoupling protein

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decreased by mild uncoupling [10,11], mitochondrial anion carrier proteins (MACPs) including uncoupling protein (UCP) and adenine nucleotide translocator (ANT) may have an inhibitory action on the overproduction of mitochondrial superoxide.

In heat stress condition, we showed that downregulation of expression of avUCP protein and mRNA was accompanied by increased mitochondrial superoxide production [12]. It can be assumed that an appropriately enhanced expression of avUCP may play a role in alleviation of mitochondrial superoxide production under conditions of acute heat stress. However, the role of avUCP in controlling superoxide production under heat stress is not well known, though its role during cold stress [13,14] and fasting [15,16] having been extensively studied.

In order to provide details on the mechanistic changes in superoxide anion production in heat-stressed chickens, therefore, we studied progressive and sequential changes in skeletal muscle mitochondrial superoxide production, expression of avUCP and avANT, and mitochondrial substrate oxidation in chickens exposed to acute heat stress.

2. Materials and methods

2.1. Animals and experimental design

Meat-type chicks (Cobb) 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 continuous light for one week, and provided with ad libitum access to water and commercial starter meat-type chick diet. At 16 d of age, birds were exposed to high temperature, 34°C, and stored at 80°C for 10 min. The supernatant was centrifuged at 1000 × g for 10 min and then 8700 × g for 10 min. The resulting pellet, containing SS mitochondria, was suspended in buffer B containing 250 mM sucrose, 20 mM Tris base, 1 mM EDTA, pH 7.4 and then washed by centrifugation at 8700 × 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. For Western blots, mitochondria were stored at −80°C until required.

2.2. Blood NEFA analysis

Blood samples were centrifuged at 700 × g for 10 min. Plasma non-esterified fatty acid (NEFA) concentrations were quantified using the NEFA C-test (Wako Pure Chemical Industries, Osaka, Japan).

2.3. Isolation of mitochondria

Muscle subsarcolemmal (SS) mitochondria were isolated from pectoralis superficialis as previously described [18]. 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 (hydroxymethyl) aminomethane (Tris) base, 5 mM MgCl₂, 5 mM ethylene glycol-bis-(β-aminohydroxy ether)-N,N,N',N'-tetra-acetic acid (EGTA), 100 mM KCl, pH 7.4 and homogenized with a Potter-Elvehjem homogenizer (5 strokes). The homogenate was then centrifuged at 800 × g for 10 min. The supernatant was centrifuged at 1000 × g for 10 min and then 8700 × g for 10 min. The resulting pellet, containing SS mitochondria, was suspended in buffer A and re-centrifuged at 8700 × g for 10 min. Following this, the pellet was re-suspended in buffer B (containing 250 mM sucrose, 20 mM Tris base, 1 mM EDTA, pH 7.4) and then washed by centrifugation at 8700 × g for 10 min. The final SS mitochondrial pellet was suspended in a minimal volume of buffer B and kept on ice.

2.4. Mitochondrial superoxide production

The LDLC method, using a Berthold luminometer [7], was performed to measure superoxide anions produced by the mitochondria isolated from the pectoralis superficialis of control and heat-stressed chickens. The reaction conditions were as follows: 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 2.5 mM potassium phosphate, 0.5 mM EDTA, pH 7.4, 20 μM lucigenin and 0.5 mg/mL mitochondria. After recording background LDLC for 4 min, the assay was initiated by the addition of 5 mM malate and 10 mM glutamate. LDLC was recorded at 2 s intervals for 5 min and the data expressed as the area under the curve calculated by integration.

2.5. Quantitation of mRNA using real-time RT-PCR

Standard molecular biological techniques were used, essentially as described by Sambrook et al. [19]. Tissues were homogenized in Trizol-Reagent (Invitrogen, San Diego, CA) and total RNA isolated according to the manufacturer’s protocol. To study progressive alterations in the expression of skeletal muscle target genes, that is, avUCP, avANT and fatty acid oxidation-related genes (CPT-I; carnitine-palmitoyl-transferase-I; CPT-II carnitine-palmitoyl-transferase-II, 3HADH: 3-hydroxyacyl CoA dehydrogenase; and LCAD: long-chain acyl CoA dehydrogenase), real-time RT-PCR analysis was performed using the iCycler Real Time Detection System (Bio-Rad Laboratories, Hercules, CA). Five micrograms of total RNA, prepared using Trizol-Reagent (Invitrogen, San Diego, CA), was reverse transcribed using a mixture of oligo(dT)12–18 and random primers, and M-MLV reverse transcriptase (Invitrogen, San Diego, CA). One microlitre of each reverse transcription reaction product then served as a template in a 50 μL PCR reaction containing 2 mM MgCl₂, 0.5 μM of each primer and 0.5x SYBR Green master mix (Bio Whittaker 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 are shown in Table 1. 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 rRNA to correct for differences in the amounts of template cDNA used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer (5′–3′)</th>
<th>Antisense primer (5′–3′)</th>
<th>Fragment size (bp)</th>
<th>Annealing (°C)</th>
<th>GenBank® Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3HADH</td>
<td>CTggTgCCATAATgATggAgT</td>
<td>TCAATCCACCAATGCTCTGTCgT</td>
<td>230</td>
<td>65</td>
<td>CD215336</td>
</tr>
<tr>
<td>avANT</td>
<td>TATCgCATgATgATgATgACAg</td>
<td>ACAgTATgATgATgCTCTTigCTgT</td>
<td>119</td>
<td>66</td>
<td>AB088686</td>
</tr>
<tr>
<td>avUCP</td>
<td>AACTCTggtgAgddCCTCACC</td>
<td>ATgTACgCcTgTCTCACCACgATC</td>
<td>433</td>
<td>66</td>
<td>AB088685</td>
</tr>
<tr>
<td>CPT-I</td>
<td>AAggATTgAACgACgAGAggA</td>
<td>CCACgAggAggCCAAACAATAggAg</td>
<td>284</td>
<td>63</td>
<td>AY675193</td>
</tr>
<tr>
<td>CPT-II</td>
<td>gCTggTATgATgATgATgAgAggA</td>
<td>CAAgTTCCTgCTgATggAA</td>
<td>137</td>
<td>63</td>
<td>NM_001031287</td>
</tr>
<tr>
<td>CS</td>
<td>AaggATTgAACgACgACgACg</td>
<td>CACgCggTACgTACTACgTACC</td>
<td>282</td>
<td>64</td>
<td>BG712956</td>
</tr>
<tr>
<td>LCAD</td>
<td>CAgTACTgCTgACAggATgAggA</td>
<td>AggCggTACgTgCggTAACAggTA</td>
<td>136</td>
<td>65</td>
<td>XM_421661</td>
</tr>
<tr>
<td>18s rRNA</td>
<td>TaAATgATgATgATgATgACAg</td>
<td>gACTgTgACgTCTCgTgAggTg</td>
<td>312</td>
<td>63</td>
<td>AJ73612</td>
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</table>

3HADH, 3-hydroxyacyl CoA dehydrogenase; avANT, avian adenine nucleotide translocator; avUCP, avian uncoupling protein; CPT-I, carnitine-palmitoyl-transferase-I; CPT-II, carnitine-palmitoyl-transferase-II; CS, citrate synthase; LCAD, long-chain acyl CoA dehydrogenase; 18s rRNA, 18s ribosomal RNA.
2.6. Quantification of avUCP protein using Western blotting

Western blot analysis for mitochondrial avUCP was carried out as described previously [15]. Briefly, SS mitochondria isolated from the pectoralis muscle were solubilized (75 μg mitochondrial protein) in the sample buffer (1% (w/v) SDS, 60 mM Tris–HCl, pH 6.8, 10% glycerol, 1 mM dithiothreitol (DTT)) and boiled for 3 min. Electrophoresis (SDS-PAGE) was carried out using a 5% polyacrylamide stacking gel and a 14% polyacrylamide resolving gel. After separation by electrophoresis, proteins were transferred to a Pure Nitrocellulose Membrane (0.2 μm, Bio-Rad) (100 mA, 80–100 V, 1.5 h) using blotting buffer (20% methanol, 25 mM Tris–HCl, 192 mM glycine). Membranes were treated with blocking buffer (7% skimmed milk, 0.1% Tween 20 in PBS) for 1 h at 25 °C, and incubated with antibodies diluted in PBS containing 7% skim milk powder. Avian UCP proteins were detected with a polyclonal antibody recognizing avUCP [12] at a dilution of 1:5000 (incubated at 4 °C overnight). Binding specificity of the antibody was confirmed by the addition of recombinant avUCP to the PBS solution, leading to the disappearance of the specific signal in Western blot experiments. After rinsing in PBS solution containing 0.1% Tween 20, membranes were incubated (25 °C for 1 h) with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at a dilution of 1:200 (incubated at 4 °C overnight). Immune-reactive avUCP was detected semi-quantitatively with densitometer tracing using a Molecular Imager FX (Bio-Rad).

2.7. 3HADH and CS activity

3HADH and CS activities were measured according to the methods of Bradshaw and Noyes [20] and Srere [21], respectively.

2.8. Statistical analysis

Data were analyzed using the statistical analysis system [22]. Data were first analyzed by a general linear model analysis of variance procedure and the means were compared using Duncan’s least significance multiple-range test. All data are expressed in the form of means ± standard error (S.E.). Differences were considered significant for values of P < 0.05.

3. Results

3.1. Plasma NEFA concentrations

As shown in Fig. 1, the plasma NEFA concentration increased rapidly to 3-fold that of control levels after 6 h of heat stress (control chickens, 0.13 ± 0.01 mEq/l; heat-stressed chickens, 0.38 ± 0.02 mEq/l; P < 0.05). After 12 h and 18 h of heat stress the NEFA concentration decreased, although the level was still higher than that of control chickens.

3.2. Superoxide production in muscle mitochondria

The results of LDCL experiments on mitochondria isolated from the pectoralis muscle of heat-stressed chickens are shown in Fig. 2. Mitochondrial superoxide anion production, determined by oxidation of glutamate and malate, was significantly enhanced 1.7-, 1.4- and 1.7-fold after 6, 12 and 18 h of acute heat stress, respectively.

3.3. Changes in anion carrier transcript expression and mitochondrial avUCP protein content in pectoralis muscle

Fig. 3 shows heat-stress-induced alterations of avUCP and avANT mRNA expression in the pectoralis muscle, as analyzed by real-time RT-PCR. Levels of avUCP mRNA were significantly decreased (P < 0.05), to 44% of control levels after 6 h of heat stress and a further slight decrease was observed at the 12 h and 18 h of heat stress (Fig. 3A). In contrast, avANT mRNA expression did not show a statistically significant change on exposure to heat stress (Fig. 3B). We subsequently studied the modulation of avUCP protein in mitochondria using an anti-avUCP polyclonal antibody. As shown in the Western blot in Fig. 4, the avUCP protein content of SS mitochondria isolated from pectoralis muscle was not changed after 6 h of heat stress; however, it was reduced to approximately 50% of the control level after 12 h and 18 h of heat stress. This finding is interesting given that the decrease in mRNA levels seen here does not correspond to a similar decrease in protein levels.

3.4. Progressive alteration of mitochondrial capacity to transport and oxidize fatty acids

Expression of the genes related to mitochondrial fatty acid transport and oxidation (CPT-I, CPT-II, 3HADH and
Expression of CPT-II, 3HADH and LCAD genes significantly increased \( (P < 0.05) \) after 6 h of heat stress and then decreased gradually. After 12 h of heat stress, expression of these genes was similar to control levels, while after 18 h of heat stress they were even lower \( (P < 0.05) \) than control levels.

Fig 5B and C shows that 3HADH and CS activity in pectoralis muscle significantly increased \( (P < 0.05) \) after 6 h of heat stress, being about 1.5-fold higher than control levels. The activities of both decreased after 12 h and 18 h of heat exposure to levels similar to control.

4. Discussion

The findings in the present study clearly showed that ROS production in skeletal muscle was similarly enhanced regardless of exposure time (6, 12 and 18 h) to heat (34°C). We thus focused on the potential mechanism by which ROS production occurs under these conditions. Downregulation of MACPs such as UCP and ANT may enhance mitochondrial superoxide anion production in heat-stressed chickens: it has been proposed that in mitochondria a weak uncoupling could be sufficient to suppress ROS production \[0.10\]. Therefore, we hypothesized that MACPs may be key regulators of mitochondrial ROS production. In fact our previous study showed that heat-stressed broiler chickens whose mitochondrial avUCP gene expression and protein levels were downregulated produced more ROS from skeletal muscle mitochondria than control chickens \[0.12\]. In contrast, white leghorn cockerels with persistent expression of avUCP on exposure to heat stress showed no change in mitochondrial ROS production \[0.23\]. These findings suggest that avUCP may play a key regulatory role in alleviating mitochondrial ROS under heat stress conditions. However, it should also be noted that increased oxidation of substrates in mitochondria may be another possible cause of enhanced mitochondrial superoxide production in heat-stressed chickens, because superoxide and other reactive species (ROS) are thought to be produced as inevitable by-products of normal aerobic metabolism. To elucidate the sequential mechanism of increased mitochondrial superoxide production in heat-stressed chickens we studied progressive changes in the expression of MACPs and mitochondrial substrate oxidation.

First, we studied progressive changes in expression of avUCP in skeletal muscle of chickens exposed to acute heat stress of increasing duration. Our data show that avUCP gene expression was downregulated after 6 h of heat stress, while avUCP protein content was decreased only after 12 h (Figs. 3A and 4). This means that there is time lag between downregulation of avUCP gene expression and decrease in the protein content, and that enhanced ROS production may be directly involved in the downregulation of avUCP protein expression in mitochondria after 12 h and 18 h of heat stress, but not after 6 h. It cannot be completely ruled out that the former responses could be the result of the complex interplay between superoxide concentration and the membrane potential value whose changes are involved in the level of energy coupling depending on the levels of avUCP present: lucigenin is known to be a penetrating cation which electrophoretically accumulates in energized mitochondria, though the concentration of lucigenin used in this experiment (20 μM) should be non-redox cycling \[0.24\]. The findings that not only UCP but also ANT can mediate uncoupling by free fatty acids \[0.25–27\] allows us to postulate that the initial stage of increased superoxide formation in heat-stressed chickens might also be associated with suppres-
sion of ANT. In fact, Echtay et al. [28] also demonstrated that the uncoupling action of ANT as well as UCP was stimulated by 4-hydroxynonenal (HNE). It was also shown that knocking out one of two ANT isoenzymes (muscle-specific ANT1) results in a strong increase in ROS production by muscle mitochondria [29]. Therefore, we studied the possible roles of avANT in suppressing superoxide production via an uncoupling action in heat-stressed chickens, but found that the duration of heat stress did not affect avANT transcript expression (Fig. 3B). Previous studies have shown that exposure to cold stress increased expression of avUCP [18,30,31] and avANT [18] gene transcripts in the skeletal muscle of birds. These results led to the hypothesis that expression of avUCP and avANT influence thermogenesis. The present results are consistent with this previous observation because we now report that heat stress downregulates avUCP gene expression. Thus, both the cold stress and heat stress results are consistent with a proposed thermoregulatory function of avian skeletal muscle mitochondria [32], in particular of avUCP. A heat-induced decrease in the mitochondrial avUCP protein level is also consistent with the above conclusion. In contrast, the avANT gene transcript seems to be regulated differently by cold stress and heat stress, with increased levels detected on exposure to cold stress and unchanged levels in chickens exposed to acute heat stress.

Alternatively, the increased mitochondrial substrate oxidation in response to 6 h-heat stress may possibly have lead to higher mitochondrial superoxide production. To clarify this point we analyzed the expression of genes for CPT-I, CPT-II, LCAD and 3HADH, and measured the activity of 3HADH and CS enzymes in skeletal muscle (involved in the mitochondrial capacity to transport and oxidize fatty acids). Expression levels of mRNA for CPT-I and CPT-II were increased significantly after 6 h of heat stress. This increase in expression of CPTs was associated with an almost similar pattern of plasma NEFA levels, suggesting that CPTs were upregulated during the initial stages of heat stress, possibly to provide more fatty acid (FA) as an energy yielding substrate to mitochondria for support of increased mitochondrial oxidation (Fig. 5A). Gene transcript levels of mitochondrial β-oxidation pathway enzymes, LCAD and 3HADH (which act on the fatty acyl-CoAs to generate FADH₂ and NADH), and 3HADH and CS enzyme activities also showed an initial increase after 6 h of heat stress (Fig. 5A and B). After the initial increases in oxidative capacity, mRNA expression and activity of these enzymes returned to control levels. These results indicate that during the first 6 h of heat stress there is an increase in mitochondrial transportation and enhanced oxidation of FA, thus augmenting mitochondrial substrate oxidation. This increased oxidation may be responsible for overproduction of mitochondrial superoxide anion, especially after 6 h of heat stress. It should be noted that UCP expression after 6 h of heat stress might have not been large enough to alleviate overproduction of ROS, even though an endogenous FFA, a potential uncoupler, might have been present in the mitochondria.

This notation leads us to advance the following discussion on the association between UCP expression and plasma FFA: in mammals, a positive correlation of UCP-3 gene expression with plasma FFA levels, which are known to increase as a result of stored fat lysis under fasting conditions,
has been reported [33,34]. Moreover, birds maintained under physiological conditions in which an increase in fatty acid metabolism take place, such as cold exposure [18], fasting [15] and hyperthyroidism [35], exhibited increases in avUCP mRNA levels. However, our previous results with fasting [15] suggested that alterations in gene transcript levels for avUCP and enzymes in the mitochondrial \( \beta \)-oxidation pathway occurred in two time-dependent patterns, but did not indicate a correlation between avUCP, FFA levels and lipid metabolism during fasting. Samec et al. [36] also emphasized that FFA is not directly related to UCP mRNA expression. These findings are consistent with the present results showing the UCP was not upregulated even when plasma FFA was enhanced in heat-stressed birds.

In conclusion, during the initial stage of acute heat stress \( \beta \)-oxidation enzymes gene transcripts and activity were upregulated, with elevated plasma NEFA levels and increased expression of mitochondrial fatty acid transport genes. This immediate increase in mitochondrial substrate oxidation resulted in higher superoxide production that might be beyond the capacity of avUCP to control superoxides. On the other hand, during the later stages of heat stress downregulation of avUCP caused increased mitochondrial superoxide production even though at this stage substrate oxidation was returned to control levels.

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References


