Mitochondrial Oxidative Damage in Chicken Skeletal Muscle Induced by Acute Heat Stress

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Mitochondria have long been known to play a critical role in maintaining bioenergetic status under physiological conditions. Our previous studies have shown that acute heat stress increases production of mitochondrial reactive oxygen species (ROS) in the skeletal muscle of chickens. This increased ROS production may lead to nonspecific modification of lipids and proteins, which may then result in bioenergetic dysfunctions. If increased mitochondrial ROS production in heat-stressed chickens causes oxidative damage, changes in mitochondrial peroxidized lipids and oxidatively modified proteins can be detected. To study this, 3-week-old male broiler chickens (n=4–8) were exposed to acute heat stress (34°C for 18 h) while control chickens were kept at thermoneutral condition (25°C). Skeletal muscle subsarcolemmal mitochondria were isolated and used to study mitochondrial malondialdehyde (MDA) and protein carbonyl groups. Mitochondrial thiobarbituric acid reactive substances (TBARS) formation was measured colorimetrically with *butylated hydroxytoluene (BHT) and expressed as MDA equivalent. To detect oxidation-sensitive mitochondrial proteins, polypeptides resolved by two-dimensional (2D) electrophoresis were immunostained with DNP-specific antibodies for carbonylated proteins using Western blotting. In heat-stressed chickens, mitochondrial MDA was 2.7-fold higher, and 82 mitochondrial proteins were oxidized when compared to that of control chickens. These results suggest that in heat-stressed chickens, increased mitochondrial ROS production leads to oxidative damage to mitochondrial lipids and proteins.

Key words: 2D electrophoresis-protein carbonyl, heat stress, lipid peroxidation, MDA, oxidized protein


Introduction

Mitochondria have long been known to play a critical role in maintaining bioenergetic status under physiological conditions. Perturbations in the physiological function of mitochondria inevitably disturb metabolism, leading to reduction in growth and even the death of chickens. Mitochondria are pivotal in controlling life and death, not only by producing ATP and sequestering calcium, but also by generating free radicals and serving as repositories for proteins which regulate intrinsic metabolic pathways. In mitochondria, superoxide anion and other reactive oxygen species (ROS) are thought to be produced as inevitable by-products 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

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superoxide anion. While ROS may play an important role in cellular functions such as cell signaling, it has been shown that high levels of ROS also cause cellular damage (Beckman and Ames, 1998), at least at the mitochondrial level (Raha and Robinson, 2000).

In chickens, heat stress causes metabolic changes in substrate oxidation and ROS production in mitochondria. We provided direct evidence of mitochondrial superoxide generation in the skeletal muscle of heat-stressed chickens by using both electron spin resonance (ESR) spectroscopy, with 5,5-dimethyl-1-pyrroline N-oxide as a spin trap agent, and lucigenin-derived chemiluminescence (Mujahid et al., 2005). We recently reported that in heat-stressed broiler chickens, the mitochondrial avUCP contents were down-regulated, thereby possibly leading to increased mitochondrial ROS production (Mujahid et al., 2006). Enhanced ROS production in mitochondria of heat-stressed chickens may lead to non-specific modification of lipids, proteins, and nucleic acids, which then result in bioenergetic dysfunctions. Mitochondrial membrane constituents are particularly susceptible to oxidative damage by ROS. If in heat-stressed chicken increased mitochondrial ROS production causes oxidative damage, changes in mitochondrial oxidatively-modified lipids and proteins can be detected. Therefore, we studied oxidative damage to mitochondrial lipids and proteins in heat-stressed chickens. We used two markers of oxidative damage, malondialdehyde (MDA) and two-dimensional (2D) electrophoresis of protein carbonyls to elucidate mitochondrial lipid peroxidation and oxidized proteins, respectively. The major phospholipid components of the mitochondrial membranes are rich in unsaturated fatty acids that are particularly susceptible to oxygen radical attack because of the presence of double bonds, which undergo peroxidation through a chain of oxidative reactions. Quantification of protein carbonyl groups is often used as an indicator of protein oxidation (Levine et al., 1990). Identification of oxidative modifications in proteins has recently progressed substantially through the use of combined immunologic and proteomic methods (Davies et al., 1987; Amici et al., 1989; Uchida and Stadtman, 1993; Miyata et al., 1998; Requena and Stadtman, 1999). Therefore, firstly we separated proteins by 2D electrophoresis and then immunostained with an anti-dinitrophenyl (anti-DNP) antibody to visualize the oxidized proteins. Applying a combined immunologic and proteomic approach should provide useful information about possible relationships between oxidized protein and heat stress.

Materials and Methods

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 a commercial starter meat-type chick diet. At 3 weeks of age chickens were exposed to high temperature, 34°C for 18 h (humidity 55±5%) while control chickens were kept at 25°C. Chickens were sacrificed by decapitation, and pectoralis superficialis muscles were immediately excised. For isolation of mitochondria, muscles were placed in ice-cold isolation buffer A (see below). Fresh mitochondria thus isolated were stored at −80°C and were used for MDA analysis within one week. Remaining mitochondria were used for 2D electrophoresis-protein carbonyl analysis. All experiments were performed in accordance with institutional guidelines concerning animal use.

Isolation of Mitochondria

Muscle subsarcolemmal (SS) mitochondria were isolated from pectoralis superficialis tissue 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 (hydroxymethyl) aminomethane (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). 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 resulting pellet was re-suspended in buffer B (containing 250 mM sucrose, 20 mM Tris base, 1 mM EGTA, 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 and mitochondria were stored at −80°C until required.

**Mitochondrial and Plasma MDA**

Subsarcolemmal mitochondria and blood plasma were used for MDA measurements. Mitochondria were isolated from pectoralis muscle (as described above) while plasma was collected by centrifuging blood samples at 700g for 10 min. Lipid peroxidation was determined colorimetrically as 2-thiobarbituric acid reactive substance (TBARS) according to Ohkawa et al. (1979) with modifications. Mitochondrial or plasma samples were mixed with 8.1% sodium dodecyl sulfate, 20% acetic acid (pH 3.5), 0.8% 2-thiobarbituric acid and 0.8% butylhydroxytoluene. After vortexing, samples were incubated for one hour on ice and then at 95°C for one hour before being transferred to ice. Butanol-pyridine 15:1 (v/v) was added, the samples were mixed by vortexing and centrifuged at 1000g for 10 min. Absorbance of the supernatant, consisting of the butanol-pyridine layer, was measured at 532 nm. The content of TBARS is expressed as the MDA equivalent. The samples were analyzed within one week of storage at −80°C.

**2-Dimensional Protein Electrophoresis**

**Sample preparation, isoelectric focusing, and 2D-PAGE**

Mitochondrial protein (25μg) pooled from four chickens was absorbed onto duplicate 7 cm immobilized pH gradient (IPG) strips (pH 3–10 NL) in an IPG swelling tray, rehydrated for 16 h at 20°C and the strips were then isoelectrically focused on a IPG phor II (Amersham Biosciences, UK) for 0.25 Kvh (30 min), 0.5 Kvh (30 min) and 8.0 Kvh (90 min). After equilibration and alkylation for 15 min each, IPG gel strips were transferred on to second dimension mini (8×10 cm) polyacrylamide SDS-PAGE gels (12%). Protein detection was achieved using silver staining (Plus one™ Silver staining kit, Amersham Biosciences, UK). After completion of SDS-PAGE, proteins in one set of gels were treated with fixation solutions (40% ethanol, 10% glacial acetic acid) for 30 min and then for 30 min in a sensitizing solution (30% (v/v) ethanol, 25% (v/v) glutaraldehyde, 5% (v/v) sodium thiosulphate, 830 mM sodium acetate). The gels were then washed with distilled water three times for 5 min. Gels were treated with silver nitrate solution (silver nitrate (2.5% w/v) 10 ml, formaldehyde (37% w/v) 0.04 ml, in 100 ml solution). After 20 min of silver staining, gels were washed with distilled deionized H2O (DDI H2O) twice for one min and then treated with developer solution (sodium carbonate 2.5 g, formaldehyde (37% w/v) 0.02/ml in 100 ml solution). After 5 min, when clear spots had developed, the reaction was stopped by adding stopping solution (EDTA-Na2 2H2O; 1.46 g in 100 ml DDI H2O) and incubating for 10 min. The gels were washed with DDI H2O three times for 5 min each. Finally the gels were preserved by adding 10 ml of glycerol (87% w/v) and 90 ml of DDI H2O.

**Image analysis**

Gels were scanned using a flat bed scanner (Arcus II, Agfa). The 2D electrophoresis image analysis and spot detection were carried out using the Image Master™ 2D Elite computer software v 4.01c (Amersham Pharmacia, biotech, UK).

**Derivatization of protein carbonyls after 2D electrophoresis**

Proteins carbonyls were determined using a reaction of dinitrophenyl hydrazine (DNP) with carbonyl groups (aldehydes and ketones) on proteins using methods described by Keller et al., (1993) with modifications (Iqbal et al., 2004). Proteins in the duplicate gels (second set of 2D electrophoresis gels, prepared as mentioned above) were electroblotted to polyvinylidene difluoride (PVDF) membranes in a submerged system (Pumford et al., 1990) using Hoefer transfer units (Hoefer, San Francisco, CA). PVDF membranes were incubated in one part 20 mM 2,4-dinitrophenyl hydrazine in 10% (v/v) trifluoroacetic acid and two parts 12% SDS. After 15 min, 1.5 volume of 2 M Tris-base was added and membranes were further incubated for 20 min. Blots were developed by incubating in a 1:5,000 dilution of anti-dinitrophenyl antiserum (Sigma Chemicals Co., St. Louis, MO) for 90 min. They were then washed with detergent buffer (0.5% casein, 150 mM NaCl, 10 mM Tris, 0.02% Thimerosal, 0.1% SDS, 5% Triton X-100) and twice with washing buffer (0.5% casein, 150 mM NaCl, 10 mM Tris, 0.02% Thimerosal) for 5 min each, and rinsed with DDI H2O (3 times) before and after incubation with detergent or washing buffers. Blots were in-
cubated for 90 min with anti-rabbit IgG antisera (Dako Co., Carpinteria, CA) peroxidase-labeled secondary antibodies and then washed extensively with detergent, washing buffers, and Tris-saline. The blots were then treated with substrate (Super Signal West Dura Extended Duration - Pierce, Rockford, IL) for 5 min, and the resultant chemiluminescence spots were detected using a charge-coupled device (CCD) camera (Fuji LAS 1000 plus - Fuji Photo Co., Ltd., Tokyo, Japan). Spots were quantified using Image Master™ 2D Elite software v 4.01 c (Amersham Pharmacia, biotech, UK).

**Statistical Analyses**

Data were analyzed using the Statistical Analysis System (Cary, NC). Differences between heat-stressed and control groups were assessed using the Student’s t-test for unpaired data. All data are expressed in the form of mean ± standard error (SE). Differences were considered significant when \( p < 0.05 \).

**Results**

**Mitochondrial and Plasma Malondialdehyde (MDA) Analysis**

To study oxidative damage to mitochondrial lipids in heat-stressed broiler chickens, mitochondrial MDA, an oxidative stress biomarker for peroxidized lipids, was measured. Exposure to heat stress (34°C for 18 h) resulted in a 2.7-fold higher mitochondrial MDA level (Fig. 1) than in control chickens, indicating elevated mitochondrial lipid peroxidation in heat-stressed chickens. In heat-stressed chickens the plasma MDA level was also higher (2.1-fold) than in control chickens (Fig. 2).

**Protein Carbonyl Analysis**

Mitochondrial proteins were resolved in the first dimension on IPG gel strips in the pH 3-10NL range and homogeneous 12% SDS-PAGE in the second dimension. Following silver staining and computer image analysis, polypeptides were detected in isolated mitochondria. To visualize the critical oxidation-sensitive proteins, polypeptides resolved by 2D electrophoresis were probed for carbonyl groups. Figure 3A shows the immunostained protein profile on the Western blot after staining for carbonyl groups, a marker for oxidized proteins (right) and total protein silver stains (left) from control chickens. Figure 3B shows the immunostained protein profile on the Western blot after probing carbonyl groups to detect oxidized protein (right) and total protein silver stains (left) from heat-stressed chickens. In heat-stressed chickens, 82 mitochondrial proteins were oxidized when compared to that of control chickens and most of them ranged from MW 24 to 108 kDa. Out of these proteins three polypeptide were heavily oxidized, the MW (kDa)
and PI (pH) of these polypeptides were 108, 5.4; 86, 4.2; and 23.7, 8.8, respectively.

**Discussion**

Mitochondria have received considerable attention as a principal source and target of ROS. We recently showed that mitochondrial superoxide production in heat-stressed broiler chickens was significantly increased compared to that of control chickens (Mujahid *et al.*, 2005). More recently we reported that exposure of young white leghorn cockerels to heat stress stimulates mitochondrial superoxide production, possibly via down-regulation of avian uncoupling protein (avUCP). Chicks on the other hand, with persistent avUCP expression, are better adapted to high temperature (Mujahid *et al.*, 2007). Thus appropriate expression of avUCP may play a role in alleviation of mitochondrial superoxide production and could help chickens to adapt to oxidative stress resulting from acute heat stress. 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. It was suggested that elevation of mitochondrial membrane potential due to defects in UCPs could be a cause of increased superoxide production, and mild uncoupling mediated by UCP could be sufficient to suppress

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**Fig. 3.** Oxidatively modified mitochondrial proteins visualized by two-dimensional (2D) electrophoresis in control (A) and heat-stressed chickens (B). Mitochondrial protein was subjected to isoelectric focusing (7-cm IPG strips, pH 3–10NL) and proteins were subsequently separated by SDS-PAGE. 2D electrophoresis of mitochondria isolated from pectoralis superficialis muscles of control and heat-stressed chickens were silver stained or immunostained to visualize normal and oxidized proteins, respectively (samples were pooled from four birds).
mitochondrial superoxide production (Skulachev, 1998; Brand et al., 2004). In fact, in heat-stressed chicken whose avUCP gene expression and protein levels in mitochondria were down-regulated, more superoxide was produced from skeletal muscle mitochondria compared with control chickens (Mujahid et al., 2006, 2007). The chickens showing down-regulation of avUCP and high ROS production in their mitochondria may have higher oxidative damage. Therefore, we studied oxidative damage to mitochondrial lipids and proteins in heat-stressed chickens.

Of the many biological targets of oxidative stress, lipids are the most common. Malondialdehyde (MDA) is the principal product of polyunsaturated fatty acid peroxidation. In the present study we found a significant increase in mitochondrial and plasma MDA of heat-stressed chickens compared to that of control chickens. Lipoperoxidation of mitochondrial membranes can be considered not only as a detoxification reaction, but also as a new source of radicals due to the self-propagating nature of the highly reactive radicals. Lipoperoxidation starts by formation of carbon-centered radicals (RC • R) at the “iso-prostanoid” units of unsaturated (ideally polyunsaturated) fatty acid chains of phospholipids and other lipid components (Spiteller, 2002). Oxidation of RC • R with O2 produces peroxyl radicals (ROO ’) that react with the nearby polyunsaturated fatty acid (PUFA)-side chain (Broekemeier et al., 2002; Guidarelli and Cantoni, 2002; Taketo and Sonoshita, 2002; Williams and Gottlieb, 2002; Nakashima et al., 2003). These reactions yield hydroperoxide side chains (ROOH, i.e. PLOOH) or free fatty acid hydroperoxides (FAOOH) and other RC • R, and this lipoperoxidation is a self-propagating reaction within the membrane (Spiteller, 2002). The main source of MDA in biological samples is the peroxidation of polyunsaturated fatty acids with two or more methylene-interrupted double bonds. Therefore, the high MDA levels observed in this study suggest that mitochondrial and plasma lipids are heavily peroxidized in heat-stressed chickens.

Proteomics combined with immunological methods for protein carbonyl detection is considered to be a powerful tool for the detection of oxidized proteins. To determine oxidative damage to mitochondrial proteins, 2D electrophoresis and detection of carbonyl groups were carried out. Large numbers of mitochondrial proteins in this study exhibited increased oxidative damage in heat-stressed broiler chickens. The oxidative damage of these mitochondrial proteins is probable due to the increased mitochondrial ROS production induced by acute heat stress (Mujahid et al., 2005), suggesting a critical role of oxidized proteins in the pathogenesis of heat stress. In the past, studies on the pathophysiology of heat stress have focused on the physiological aspects, levels of antioxidant enzymes, heat shock proteins or lipid oxidation. In contrast, our approach was to study not only peroxidation of lipids but also oxidative damage of mitochondrial proteins due to increased ROS production under acute heat stress conditions. This approach was aimed at providing additional insight into the understanding of the pathogenic mechanism of acute heat stress at the mitochondrial level. Although it was clear from this study that mitochondrial proteins are oxidized due to acute heat stress, it is not yet clear to what extent the various biological properties of these proteins are compromised, altered, or destroyed. Obviously such modifications have potentially wide-ranging effects on protein function (e.g., catalytic activity, substrate binding, stability), leading to inefficient metabolism in mitochondria. Clearly, identifying these particular ROS-sensitive proteins and elucidating the consequences of these modifications in mitochondria of heat-stressed chickens is of major interest and should be the topic of future studies.

In conclusion, this study has shown oxidative damage resulting from heat stress in chickens affects mitochondrial lipids and a large number of mitochondrial proteins, which coincides with previously demonstrated down-regulation of avUCP levels and up-regulation of ROS production.

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