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Membrane potential and H_2O_2 production in duodenal mitochondria from broiler chickens (*Gallus gallus domesticus*) with low and high feed efficiency

C. Ojano-Dirain [a,*], N.B. Tinsley [a], T. Wing [b], M. Cooper [b], W.G. Bottje [a]

[a] *Department of Poultry Science, Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA*
[b] *Cobb-Vantress, Inc., Siloam Springs AR 72761-1030, USA*

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Abstract

Increased hydrogen peroxide (H_2O_2) production was observed in duodenal mitochondria obtained from broiler chickens with low feed efficiency (FE). As a decrease in mitochondrial membrane potential ($\Delta\psi_m$) due to mild uncoupling of oxidative phosphorylation reduces reactive oxygen species production, this study was conducted to evaluate the effect of uncoupling on $\Delta\psi_m$ and H_2O_2 production in duodenal mitochondria isolated from broilers with low (0.48±0.02) and high (0.68±0.01) FE. Membrane potential and H_2O_2 production were measured fluorometrically and in the presence of different levels of an uncoupler, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). The $\Delta\psi_m$ was higher ($P \leq 0.05$) in high FE mitochondria at 0 to 600 nM FCCP. A decrease in $\Delta\psi_m$ was observed at 600 and 1000 nM FCCP in the low and high FE groups, respectively. H_2O_2 generation was higher in the low FE mitochondria at all FCCP levels except at 200 nM. Adding 200 to 800 nM FCCP decreased H_2O_2 production in low but not in high FE mitochondria. These results showed that FCCP-induced uncoupling lowered H_2O_2 production in low FE but not in high FE duodenal mitochondria and suggest that $\Delta\psi_m$ may influence H_2O_2 production in low FE mitochondria.

Keywords: Broilers; Duodenal mitochondria; Feed efficiency; H_2O_2 production; Membrane potential

1. Introduction

Feed cost represents 50 to 70% of the cost of producing livestock. Thus, high feed efficiency (FE, gain:feed) is a major selection pressure placed on commercial broilers (meat-type chickens) to help lower production cost and increase profitability of broiler production. Modern broiler strains have a remarkable improvement in FE (Havenstein et al., 2003) but there remains about 10% variation within broiler lines heavily selected for FE (Emmerson, 1997). Studies in broilers (Bottje et al., 2002, 2004; Iqbal et al., 2004, 2005; Ojano-Dirain et al., 2004, 2005a,b), rats (Lutz and Stahly, 2003), cattle (Sandelin et al., 2004) and in swine (Feliz et al., 2005) provided evidence that variations in phenotypic expression of FE, without breed and dietary effect, might be explained in part by differences in mitochondrial function and biochemistry.

Mitochondria are the main site of energy (ATP, adenosine triphosphate) production in eukaryotic cells. However, mitochondria are also a major source of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$). The Mn isoform of superoxide dismutase (SOD) found in mitochondrial matrix converts $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2), which can then be converted to the highly reactive hydroxyl radicals ($OH^{\cdot}$) via the Fenton reaction (Chance et al., 1979; Miwa et al., 2003). ROS are considered inevitable by-products of mitochondrial respiration resulting from univalent reduction of O_2 by electrons that leak from the electron transport chain (Chance et al., 1979). Under normal

Abbreviation: AA, antimycin A; ADP:O, adenosine diphosphate to oxygen ratio; DCF, 2′, 7′-dichlorofluorescein; DCFH-DA, 2′, 7′-dichlorofluorescin diacetate; EGTA, ethylene glycol-bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′ tetraacetic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; FE, feed efficiency; HEPES, *N*-[2-hydroxyethylpiperizine]-*N*′-[2-ethanesulfonic acid]; H_2O_2, hydrogen peroxide; $\Delta\psi_m$, mitochondrial membrane potential; Myx, myxothiazol; $O_2^{\cdot-}$, superoxide; RCR, respiratory control ratio; ROS, reactive oxygen species; SOD, superoxide dismutase; TTFA, thenoyltrifluroacetone.

* Corresponding author. Tel.: +1 479 575 5700; fax: +1 479 575 7139.
E-mail addresses: cojano@uark.edu (C. Ojano-Dirain), wbottje@uark.edu (W.G. Bottje).

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conditions, ROS are neutralized by an array of antioxidants, protecting the cell against oxidative damage. Low levels of ROS are required for normal cell functions, i.e., cell signaling (Giulivi and Oursler, 2003), but elevated ROS production can cause oxidative stress and cellular damage (Yu, 1994). Previous studies in breast muscle, liver and duodenal mitochondria isolated from low FE broilers exhibited higher rates of H_2O_2 production and higher protein oxidation compared to mitochondria from their age-matched high FE birds (Bottje et al., 2004; Iqbal et al., 2004, 2005; Ojano-Dirain et al., 2004, 2005b). These observations indicate that inefficiencies in mitochondrial function such as increased H_2O_2 production and/or higher protein oxidation may be responsible in part for the poorer FE in low FE broilers.

Proton motive force (Δp) is the redox energy created by the translocation of protons from the matrix into the intermembrane space in the electron transport chain and drives ATP synthesis from ADP and inorganic phosphate by ATP synthase (Lehninger et al., 1993). Proton motive force has two components, a membrane potential ($\Delta\psi$) and a pH gradient (ΔpH). Skulachev (1998) suggested that dissipation of the membrane potential, by transporting proton back into the matrix via uncoupling proteins, could attenuate ROS production. In studies with Drosophila mitochondria, Miwa et al. (2003) also demonstrated that mild uncoupling of oxidative phosphorylation with a chemical uncoupler, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), decreased $O_2^{\cdot-}$ production. To our knowledge, the effect of uncoupling on H_2O_2 production in broiler mitochondria has not been investigated. Since mitochondrial dysfunction and energetic inefficiency in the gastrointestinal tract could contribute to the phenotypic expression of low FE, this study was conducted to: 1) evaluate the effect of FCCP on membrane potential and H_2O_2 production, and 2) determine the topology of H_2O_2 in duodenal mitochondria isolated from broiler breeder males with low and high FE.

2. Materials and methods

2.1. Birds, sampling and mitochondria isolation

Male broilers (*Gallus gallus*) with the highest or lowest 7 to 8-wk FE ($n=8$/group) were selected from a group of 100 broiler breeder replacement stock (Cobb-Vantress, Inc., Three Springs Farm, OK, USA) tested for FE as previously described (Bottje et al., 2002; Ojano-Dirain et al., 2004). Birds were color-coded and researchers were not aware of the FE data until completion of experiments. Birds were transported from a local breeder company to the University of Arkansas, where they were housed individually in similar cages (51 × 51 × 61 cm) and environmental conditions (25 °C; 15 L: 9D) and fed the same diet provided during the FE trial (20.5% protein, 3280 kcal/kg). Upon arrival at the university facility, one bird was euthanized due to a broken wing. Birds were provided free access to feed and water. After a 5-day acclimation, one bird per day was randomly selected for mitochondria isolation, with group selection alternated on each day. Birds were euthanized with an overdose of sodium pentobarbital by i.v. injection into the wing vein.

Duodenal mitochondria were isolated by differential centrifugation (Lawrence and Davies, 1986; Ojano-Dirain et al., 2004). Briefly, the mucosa was homogenized, centrifuged, and the supernatant and crude mitochondrial fraction was centrifuged for 7 min at 9800 and 12,100 ×*g*, respectively. The mitochondrial pellet was enriched (12,100 ×*g* for 7 min, twice) and resuspended in isolation medium. Mitochondrial protein was determined with a protein assay kit (Sigma Kit 610-A Sigma-Aldrich Chemical Co. St. Louis, MO, USA) as described (Lawrence and Davies, 1986; Ojano-Dirain et al., 2004). Mitochondrial protein was 2.87±0.30 and 2.64±0.20 mg/mL for high and low FE group, respectively.

2.2. Respiration rates and mitochondrial function

Respiration rates (nmol monomeric oxygen/min per mg mitochondrial protein) were determined by measuring oxygen consumption of freshly isolated mitochondria (0.2 mg protein/mL, final concentration) with a 1-mL Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH, USA) at 37 °C (Estabrook, 1967; Ojano-Dirain et al., 2004). Mitochondria were incubated in assay medium with 10 mM succinate as substrate. State 3 or active respiration was measured after ADP addition. As ADP levels become limiting following conversion to ATP, mitochondria enter State 4 or resting respiration.

The respiratory control ratio (RCR), an index of respiratory chain coupling, was calculated by dividing State 3 by State 4 respiration rate while ADP to oxygen ratio (ADP:O), an index of oxidative phosphorylation, was determined by dividing the quantity of ADP added by the amount of oxygen consumed during State 3 respiration (Estabrook, 1967). Similar to previous studies (Bottje et al., 2002; Ojano-Dirain et al., 2004), RCR and ADP:O were assessed with sequential additions of ADP (220 μM and 440 μM) to mimic a repeated demand for energy (Cawthon et al., 2001; Iqbal et al., 2001). All functional measurements were made in triplicate and were completed within 1 h of mitochondrial isolation.

2.3. Mitochondrial membrane potential and uncoupling

Mitochondrial membrane potential ($\Delta\psi_m$) was measured according to Scaduto and Grotyohann (1999) using tetramethylrhodamine methyl ester (TMRM) as a probe for membrane potential. TMRM is a lipophilic cationic, fluorescent dye that is readily sequestered by active mitochondria driven by their membrane potential (Scaduto and Grotyohann, 1999). Briefly, the assay was carried out at 37 °C in a medium (pH 7.4) containing 50 mM KCl, 10 mM HEPES, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 37 μM EGTA and 10 mM succinate. Mitochondria (0.2 mg /mL), 5 μM rotenone, 0.1 μM nigericin, and 0.3 μM TMRM were added to the reaction medium in 96-well microplate and changes in fluorescence was measured with a *Cytofluor* photofluorometer (Millipore Corporation, Bedford, MA, USA) at 530 nm excitation and 580 nm emission wavelengths. Uncoupling was accomplished by adding 0 to 1,000 nM of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and nigericin was used to dissipate pH gradient (Miwa et al.,