Gene Expression in Breast Muscle and Duodenum from Low and High Feed Efficient Broilers

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ABSTRACT This study was conducted to evaluate messenger RNA (mRNA) expression of genes that are involved in energy metabolism and mitochondrial biogenesis: avian adenine nucleotide translocator (avANT), cytochrome oxidase III (COX III), inducible nitric oxide synthase (iNOS), peroxisome proliferator-activated receptor-γ (PPAR-γ), avian PPAR-γ coactivator-1α (avPGC-1α), and avian uncoupling protein in breast muscle and duodenum of broilers with low and high feed efficiency (FE). Total RNA was extracted from snap-frozen tissues from male broilers with low (0.55 ± 0.01) and high (0.72 ± 0.01) FE (n = 8 per group). Total RNA was reverse-transcribed using oligo(dT), random primers, or both followed by real-time reverse transcription-PCR. Protein oxidation, measured as protein carbonyls, was also evaluated in duodenal mucosa. Protein carbonyls were higher in low FE mucosa in tissue homogenate and mitochondrial fraction. The mRNA expression of iNOS and PPAR-γ in the duodenum was lower in the low FE broilers, with no differences in avANT, COX III, and avPGC-1α. In contrast, expression of avANT and COX III mRNA in breast muscle was lower in low FE broilers with no differences in iNOS, PPAR-γ, and avPGC-1α. The avian uncoupling protein in breast muscle was higher in low FE birds (P = 0.068). These results indicate that there are differences in the expression of mRNA encoding for mitochondrial transcription factors and proteins in breast muscle and duodenal tissue between low and high FE birds. The differences that were observed may also reflect inherent metabolic and gene regulation differences between tissues.

Key words: feed efficiency, broiler, breast muscle, duodenum, gene expression, carbonyl

INTRODUCTION

Feed efficiency (FE, gain:feed) is considered one of the most important traits in livestock production since feed represents about 50 to 70% of the total cost of production. Over the last few years, we have conducted several studies that have focused primarily on the potential link between mitochondrial function, oxygen radical production, and protein expression and the phenotypic expression of FE in broiler chickens (Bottje et al., 2006). These studies have been conducted in broilers fed the same diet and from the same genetic line and were initiated using muscle mitochondria (Bottje et al., 2002; Iqbal et al., 2004). Subsequent studies have been carried out in liver (Iqbal et al., 2005), duodenum (Ojano-Dirain et al., 2004a,b; 2005a,b), lymphocytes (Lassiter et al., 2006), and heart (Tinsley et al., 2004). From these studies, it has been determined that in comparison to broiler breeder males with high FE, mitochondria obtained from phenotypically low FE birds exhibited higher H2O2 production associated with greater protein oxidation and lower activities of respiratory chain complexes. In some, but not in all cases, low FE mitochondria exhibited less tightly coupled electron transport chain with no difference or superior ability to carry out oxidative phosphorylation. Protein expression determined by Western blot analysis was also equal between low and high FE groups or was higher for some proteins (5 in complex III, and 1 in complex IV) in the low FE breast muscle mitochondria (Iqbal et al., 2004). Similar observations have also been obtained in the liver (Iqbal et al., 2005) and heart (Tinsley et al., 2004). On the other hand, an apparent upregulation of nuclear-encoded proteins and downregulation of mitochondrial-encoded subunits were observed in low FE duodenal mitochondria (Ojano-Dirain et al., 2005b). The protein expression of adenine nucleotide translocator-1 (ANT-1) was also higher in low FE breast muscle mitochondria (Iqbal et al., 2004) but was not different between high and low FE groups in the liver (Iqbal et al., 2005) and duodenum (Ojano-Dirain et al., 2005b). Thus, defin-
ing the mechanisms involved in compromised mitochondrial function in low FE broilers is unclear at this point. A key to understanding the differences in mitochondrial function and biochemistry may lie in the direction of upstream regulators of mitochondrial protein expression and biogenesis.

Nisoli et al. (2003) provided evidence that differences in mitochondrial protein expression may be due to nuclear regulation that included induction of peroxisome proliferator-activated receptor γ (PPAR-γ) and PPAR-γ coactivator-1α (PGC-1α) that in turn upregulate 2 other transcription factors, nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A. Several studies also suggest that increased reactive oxygen species (ROS) like H₂O₂ may alter gene expression (e.g., Greiber et al., 2002; Li et al., 2002; Kemp et al., 2003). As we have consistently observed greater H₂O₂ production and protein oxidation in low FE birds, it is possible that increased ROS in combination with alterations in expression of transcription factors (e.g., PPAR-γ and PGC-1α) may be critical determinants of cellular function associated with the phenotypic expression of feed efficiency in broilers. Thus, this study was conducted to evaluate mRNA expression of genes that are involved in mitochondrial energy metabolism [avian ANT (avANT), cytochrome oxidase III (COX III), and avian uncoupling protein (avUCP)] and mitochondrial biogenesis [PPAR-γ, avPGC-1α, and inducible nitric oxide synthase (iNOS)] in breast muscle and duodenal tissue obtained from broiler breeders with low and high FE.

MATERIALS AND METHODS

Birds and Sampling

Seven-week-old male broilers with the highest or lowest 6- to 7-wk FE (n = 8/group) were selected from a group of 100 breeder replacement stock (Cobb Vantress Inc., Three Springs Farm, OK) tested for FE as previously described (Bottje et al., 2002). The birds in the present study were sampled on site at the breeder company. Birds were euthanized with i.v. injection of pentobarbital into the caudal tibial vein. Breast muscle samples were collected from similar sites of breast muscle from each bird (Iqbal et al., 2004) and mucosa from the duodenal loop was also collected (Ojano-Dirain et al., 2004a). Breast muscle and mucosa samples for RNA assay were collected and frozen in liquid nitrogen within 2 min after birds were killed. Aliquots were also collected and snap-frozen in liquid nitrogen for other biochemical assays, i.e., protein carbonyls. Samples were stored at −80°C. The weight, length, and diameter of the duodenal loop and mucosa weight were also recorded.

Mucosa Homogenate and Mitochondria Isolation

Duodenal mitochondria were isolated by differential centrifugation (Lawrence and Davies, 1986; Ojano-Dirain et al., 2004a). Briefly, 1 g of mucosa was homogenized and centrifuged at 750 × g. The pellets containing nuclei and cell debris were discarded, and a portion of the homogenate was frozen in liquid nitrogen and stored at −80°C. The supernatant and the resulting crude mitochondrial fraction were centrifuged for 7 min at 9,800 and 12,100 × g, respectively. The enriched mitochondrial pellet (12,100 × g for 7 min, twice) was resuspended in isolation medium and stored at −80°C. All procedures were carried out at 4°C. A Bradford assay (Sigma Kit 610-A, Sigma Chemical Co., St. Louis, MO) was used to determine homogenate and mitochondrial protein concentration. Homogenate protein was 4.8 ± 0.2 and 4.9 ± 0.2 mg/mL for the high and low FE groups, respectively. Mitochondrial protein values were 12.9 ± 0.7 and 13.1 ± 0.8 mg/mL for the high and low FE groups, respectively. Thus, the quality of mitochondrial preparation was considered similar between groups.

Determination of Protein Oxidation (Protein Carbonyls)

Protein oxidation (protein carbonyls) was determined in duodenal mucosa homogenate and mitochondrial fractions based on a reaction of dinitrophenyl (DNP) hydrazine with carbonyl groups on proteins (Keller et al., 1993; Ojano-Dirain et al., 2005b). Briefly, total proteins (25 and 35 μg/lane for homogenate and mitochondrial fraction, respectively) were separated by SDS-PAGE in 10% polyacrylamide gels and then transferred to polyvinylidene difluoride membrane overnight. The membranes were stained with Ponceau S Staining Solution (Sigma Chemical Co.) to visualize protein transfer, following the manufacturer’s recommendations. To derivatize protein carbonyls, the membranes were incubated in 10 mL of 20 mM 2, 4-DNP in 10% (vol/vol) trifluoroacetic acid, and 20 mL 12% SDS. After 15 min, 15 mL of 2 M Tris-base was added and incubated for 20 min. For DNP immunostaining, the membranes were incubated overnight at 4°C with anti-DNP antibody (Sigma Chemical Co.), followed by incubation with secondary antibody (Pierce Biotechnology Inc., Rockford, IL). Membranes were washed with appropriate buffers before and after antibody incubations. The membranes were developed with a chemiluminescence substrate (SuperSignal West Dura Extended Duration Substrate, Pierce Biotechnology Inc., Rockford, IL), and bands were detected with a charge-coupled device camera (Fuji LAS 1000, Fuji Photo Co., Ltd., Tokyo, Japan) and quantified with Scion software (http://www.scioncorp.com). The membranes were washed and reincubated with mouse antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International, Temecula, CA) for the immunological detection of GAPDH. The GAPDH intensity was used to normalize protein loading on each of the gels. Therefore, the protein carbonyl values were calculated as the ratio of protein carbonyl to GAPDH staining intensity. Representative examples of protein carbonyl staining and GAPDH stain-
Carbonyl immunoblot of DL mucosa homogenate

GAPDH immunoblot of DL mucosa homogenate

Figure 1. Representative Western blots illustrating A) protein carbonyl staining of duodenal loop (DL) mucosa homogenate, and B) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) staining of duodenal mucosa homogenate. Lanes marked L and H indicate samples from broilers with low and high FE, respectively. MWM indicates molecular weight markers (which are not clearly visible because the blot image was developed using a chemiluminescence detection system).

Isolation of Total RNA

Total RNA was extracted from 30 mg breast muscle or mucosa using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA) according to the manufacturer’s protocol. Briefly, tissues were disrupted and homogenized with a rotor-stator homogenizer in Buffer RLT containing β-mercaptoethanol. The tissue lysate was centrifuged for 3 min at 13,200 rpm in a microcentrifuge. The supernatant was collected, added with 1 volume of 70% ethanol and mixed immediately. Each sample (700 μL) was applied to the RNeasy mini column, centrifuged for 15 s at 10,000 rpm, and the flow-through material was discarded. On-column DNase digestion was carried out using Qiagen’s RNase-Free Dnase Set. Next, the RNeasy columns were transferred to new 2-mL collection tubes, and 500 μL of buffer RPE was pipetted onto the RNeasy column followed by centrifugation for 15 s at 10,000 rpm. To dry the silica membrane, another 500 μL of Buffer RPE was pipetted onto the RNeasy column and centrifuged for 2 min at 10,000 rpm. Total RNA was eluted in 50 μL of RNase-free water. All steps were carried out under RNase-free conditions. The quality of isolated total RNA was estimated from the $A_{260\,\text{nm}}/A_{280\,\text{nm}}$ absorbance spectra (values of 1.9 to 2.1 in 10 mM Tris-HCl, pH 7.5, were considered acceptable) and by examination of the 18S and 28S bands in 1% agarose gel stained with ethidium bromide, according to the RNeasy Mini Handbook. The RNA concentration was determined by measuring the absorbance at 260 nm.

Quantitative Reverse-Transcription-PCR

Gene expression of avANT, COX III, avUCP, iNOS, PPAR-γ and avPGC-1α was assessed by 2-step quantitative real time reverse transcription-PCR (RT-PCR) following the SYBR Green PCR Master Mix and RT-PCR protocol (Applied Biosystems). Five micrograms of total RNA was reverse transcribed using a mixture of Oligo(dT)$_{12\,\text{-18}}$ primers and random primers, and Superscript II reverse transcription (Invitrogen) as described previously (Abe et al., 2006). A negative control (minus Superscript II reverse transcription) was also run simultaneously. Agarose gel electrophoresis and ethidium bromide staining checked the purity of PCR products. The cDNA samples were stored at −20°C.

Amounts of mRNA were then quantified by real-time quantitative RT-PCR using the ABI Prism 7700 Sequence Detection System and SYBR Green Master Mix Kit (Applied Biosystems). Aliquots of cDNA were subjected to PCR amplification using Qiagen’s Taq DNA polymerase and gene-specific primers. Oligonucleotide sequences of sense and antisense primers and annealing temperatures are shown in Table 1. All primer concentrations were optimized prior to actual runs. All measurements were carried out in triplicate, and the average values were obtained. A standard curve was created with serial dilutions and gene expression was quantified from the standard calibration curves run simultaneously with the samples. All quantifications used GAPDH mRNA as the internal control, and a negative control (no sample) was also used for each primer set.

The values were normalized with mRNA expression of GAPDH and expressed as the ratio of the GAPDH mRNA values in arbitrary units.
Table 1. Sequences for real-time PCR primers

<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 number</th>
</tr>
</thead>
<tbody>
<tr>
<td>avUCP</td>
<td>GCAGGCCGCAAGTGAGCTTT</td>
<td>AGAGCTGTTCACAGAGATCGTGA</td>
<td>41</td>
<td>60</td>
<td>AB088685</td>
</tr>
<tr>
<td>avANT</td>
<td>TTGTGCTTTGTGCTTTCCTTA</td>
<td>GGTCTTGACTGATCTTACCA</td>
<td>67</td>
<td>60</td>
<td>AB088686</td>
</tr>
<tr>
<td>nOS</td>
<td>TGGAGAAAGAAGCTATTCCTCAT</td>
<td>ATGTTAAACAGACTGAGATTC</td>
<td>76</td>
<td>60</td>
<td>U45604</td>
</tr>
<tr>
<td>PPARγ</td>
<td>TGGAGAATCTCATATCGCCATCA</td>
<td>TCTGGCAAGGCTTCTCTCT</td>
<td>42</td>
<td>60</td>
<td>AAF80170</td>
</tr>
<tr>
<td>avPGC-1α</td>
<td>CCAAAAGGACAGCCGCTTAGAT</td>
<td>GGCCGGAGAATAATGCGAAGA</td>
<td>76</td>
<td>60</td>
<td>AB170013</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGAAATGCCTGGCTCAACGGATT</td>
<td>CCACCTTGGAACCTTGCCAGAGA</td>
<td>81</td>
<td>60</td>
<td>NM_204305</td>
</tr>
<tr>
<td>1avUCP = avian uncoupling protein; avANT = avian adenine nucleotide translocator; iNOS = inducible nitric oxide synthase; PPAR-γ = peroxisome proliferator-activated receptor-γ; avPGC-1α = avian PPAR-γ coactivator-1α; COX III = cytochrome c oxidase subunit III; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistical Analyses**

Data were analyzed with JMP 5.0 statistical software (SAS Institute Inc., Cary, NC). Multiple comparisons were assessed with 1-way ANOVA, and means were separated by Student’s t-test. Data are presented as the mean ± SEM, and a probability level of P ≤ 0.05 was considered significant unless specified otherwise.

**RESULTS AND DISCUSSION**

Studies in our laboratory have consistently shown that mitochondria or tissues obtained from low FE male broiler breeders exhibited higher hydrogen peroxide (H2O2) production and greater protein oxidation (carbonyls). Functional and biochemical studies suggested that the electron transport chain may be less tightly coupled in low FE mitochondria, but the ability to carry out oxidative phosphorylation was not different or superior to that in high FE mitochondria (Bottje et al., 2006). Additionally, it appears that mitochondria in low and high FE birds may function differently in different tissues. Tissue specificity has also been observed in mitochondrial diseases, where only some tissue will be affected even if a defect in oxidative phosphorylation is present in all tissues (Rossignol et al., 2000). To this point, defining the cellular basis of compromised mitochondrial function in low FE broilers is still unclear. Ongoing studies in our laboratory are aimed to increase our understanding of mitochondrial physiology and phenotypic expression of FE in broilers. In the studies conducted to date, COX III and cyt c appear to be differentially expressed with broiler FE phenotype from several tissues (Iqbal et al., 2004, 2005; Lassiter et al., 2006; Ojano-Dirain et al., 2005a,b).

It is possible that differences in mitochondrial protein expression may be due to various upstream nuclear transcription regulators such as PPAR-γ and PGC-1α (Nisoli et al., 2003). Moreover, ROS such as H2O2 can regulate or alter gene expression (e.g., Crawford et al., 1997; Carper et al., 1999; Greiber et al., 2002; Li et al., 2002; Kemp et al., 2003). Several studies have shown that severe oxidative stress can upregulate expression of stress-response genes, whereas moderate oxidative stress (i.e., noncytotoxic) can specifically downregulate gene expression (Morel and Barouki, 1999). Thus, the goal of the current study is to evaluate differences in expression of a few selected genes involved in mitochondrial energy metabolism and biogenesis in broilers with low and high FE. To assess potential variations among tissues, we utilized breast muscle (a major contributor to energy utilization in the body) and duodenal mucosa (nutrient absorption and transport) in the current study.

Growth performance data are consistent with our previous studies (Bottje et al., 2002; Iqbal et al., 2004, 2005; Ojano-Dirain et al., 2004a,b, 2005a,b; Lassiter et al., 2006) wherein high FE birds gained more weight at similar feed intake compared with the low FE birds (Table 2). The absence of differences in duodenal physical measurements (Table 3) is similar to previous observations (Ojano-Dirain et al., 2004a,b, 2005a,b).

Protein oxidation, measured by the appearance of carbonyl groups (ketones and aldehydes) in proteins, is the best measure of oxidative stress (Keller et al., 1993; Stadtman and Levine, 2000). Hence, immunological detection of protein carbonyls was used to measure the level of oxidized proteins in duodenal mucosa. Similar to observations in previous studies (Iqbal et al., 2004; Ojano-Dirain et al., 2005b; Lassiter et al., 2006; Bottje et al., 2006), total protein carbonyls were higher (P ≤ 0.05)

<table>
<thead>
<tr>
<th>Variable</th>
<th>High FE (n = 8)</th>
<th>Low FE (n = 8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6- to 7-wk BW gain, g</td>
<td>793 ± 36</td>
<td>546 ± 28</td>
<td>0.001</td>
</tr>
<tr>
<td>Feed intake, g</td>
<td>1,091 ± 41</td>
<td>982 ± 44</td>
<td>0.09</td>
</tr>
<tr>
<td>FE (gain to feed), g:g</td>
<td>0.72 ± 0.01</td>
<td>0.55 ± 0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>FCR2 (feed to gain), g:g</td>
<td>1.38 ± 0.01</td>
<td>1.80 ± 0.03</td>
<td>0.001</td>
</tr>
</tbody>
</table>

1Values are mean ± SEM of values shown in parentheses.
2Feed conversion ratio.
Table 3. Duodenum loop (DL) and physiological data of broilers with low and high feed efficiency (FE)\(^1\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>High FE (n = 8)</th>
<th>Low FE (n = 8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL weight, g</td>
<td>33 ± 2</td>
<td>35 ± 1</td>
<td>0.50</td>
</tr>
<tr>
<td>DL diameter, cm</td>
<td>0.65 ± 0.02</td>
<td>0.71 ± 0.04</td>
<td>0.13</td>
</tr>
<tr>
<td>DL area, cm(^2)</td>
<td>101 ± 3</td>
<td>105 ± 4</td>
<td>0.42</td>
</tr>
<tr>
<td>Mucosa weight, g</td>
<td>9.2 ± 0.3</td>
<td>9.9 ± 0.4</td>
<td>0.13</td>
</tr>
<tr>
<td>Homogenate protein, mg/mL</td>
<td>4.8 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>0.63</td>
</tr>
<tr>
<td>Mitochondrial protein, mg/mL</td>
<td>12.9 ± 0.7</td>
<td>13.1 ± 0.8</td>
<td>0.88</td>
</tr>
</tbody>
</table>

\(^1\)Values are mean ± SEM of values shown in parentheses.

in low FE duodenal mucosa homogenate and duodenal mitochondria (Figures 2 and 3, respectively). In addition to total protein carbonyls, band-specific carbonyls, representing different ranges of molecular weights, were also analyzed. We found that all 6 protein carbonyl bands in the duodenal mitochondria were higher \((P \leq 0.05)\) in low FE compared with the high FE group. In contrast, 1 of 3 protein carbonyl bands (band 2, Figure 1A) in duodenal mucosa homogenate was not different between the high and low FE birds (Figure 2). This indicates that not all proteins in the mucosa homogenate or cytosol are equally susceptible to oxidative damage and may reflect the fact that the cell has a more dynamic antioxidant system than mitochondria (Griffith and Meister, 1985). It would be of considerable interest to determine which proteins were oxidized and which proteins were not, as well as to answer the question why or how these proteins resisted oxidation.

Greater protein oxidation in mitochondria may be responsible for lower respiratory chain complex activities in muscle, liver, and duodenal tissues (Bottje et al., 2002; Iqbal et al., 2004; Ojano-Dirain et al., 2005b). Increased oxidized proteins in low FE duodenal mitochondria may also be linked to an altered antioxidant defense mechanism (Ojano-Dirain et al., 2005a). Therefore, the higher levels of oxidized proteins in the low FE birds may contribute to the phenotypic expression of low FE by increasing energy demands on the cell to repair damaged proteins and through compromised or decreased function of damaged proteins. Elevated protein oxidation in tissues and mitochondria remains the most consistent finding we have observed with the phenotypic expression of low FE (Bottje et al., 2006).

Higher amounts of H\(_2\)O\(_2\) production in low FE broilers with subsequent protein damage may also alter mitochondrial gene expression (e.g., Crawford et al., 1997; Carper et al., 1999; Greiber et al., 2002; Kemp et al., 2003). Thus, quantitative RT-PCR was used to evaluate gene expression in breast muscle and duodenum obtained from low and high FE birds. Gene expression level of avANT, avUCP, COX III, iNOS, PPAR-\(\gamma\), and PGC-1\(\alpha\) were normalized to the GAPDH mRNA levels in the duodenum (Figure 4) and breast muscle (Figure 5) because we confirmed that the GAPDH mRNA levels normalized to 18s rRNA were not different between the high and low FE broilers in both tissues.

Adenine nucleotide translocator (ANT) or adenosine diphosphate/adenosine triphosphate (ATP) translocase is responsible for the exchange of cytosolic adenosine diphosphate for mitochondrial matrix ATP across the inner mitochondrial membrane. The ANT also participates in the mitochondrial permeability transition, a sud-
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Figure 4. The mRNA expression of avian adenine nucleotide translocator (avANT) and cytochrome oxidase III (COX III) in duodenal mucosa obtained from broilers with high and low feed efficiency (FE). The mRNA values (in arbitrary units, AU) are expressed as ratio of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA values. Each bar represents the mean ± SEM for high and low (n = 7/group) FE birds.

den increase in the inner membrane permeability that could lead to cell death by apoptosis or necrosis (Petit et al., 1996; Scheffler, 1999). Mammalian ANT has 3 isoforms: ANT-1, ANT-2, and ANT-3. The ANT-1 has been reported to exert a key metabolic control over mitochondrial energy production (Malgat et al., 2000). Avian ANT has also been identified, and avANT mRNA expression was affected by fasting and cold-exposure (Toyomizu et al., 2002, 2006). In the present study, mRNA levels of avANT were lower in breast muscle of low FE broilers (Figure 5). This contrasts to previous observations that reported higher protein expression of ANT-1 in low FE breast muscle (Iqbal et al., 2004), which could be attributed to the fact that mRNA levels and protein expression do not always coincide due to other processes such as mRNA stability and posttranslational modification (Day and Tuite, 1998). On the other hand, avANT mRNA levels in the duodenum were not different between the high and low FE birds (Figure 4), which concurs with previous findings in ANT-1 protein expression in the duodenum (Ojano-Dirain et al., 2005b) and liver (Iqbal et al. 2005).

Cytochrome c oxidase subunit III is a mitochondrial-encoded subunit in complex IV of the respiratory chain. The COX III is responsible for modulating proton pumping and electron transport through the redox centers, and together with subunits I and II, form the functional core of Complex IV (Scheffler, 1999). Therefore, COX III may play a key role in energy production. The COX III mRNA levels were not different in the duodenum of high and low FE birds (Figure 4). The lack of significant differences in gene expression of avANT and COX III in the duodenum supports our earlier observation that ATP production is not compromised in duodenal mitochondria of low FE birds (Ojano-Dirain et al., 2004a). In contrast to the duodenum, COX III mRNA levels in breast muscle were lower in low FE compared with the high FE birds (Figure 5). The lower expression of COX III mRNA in low FE breast muscle may be due to a higher oxidative damage (Iqbal et al., 2004). This hypothesis would be supported by the observations that when values were expressed as a percentage of high FE birds, total carbonyls were 75% higher in the low FE breast muscle mitochondria (Iqbal et al., 2004), whereas in the duodenum, total carbonyls were only 12% higher in low FE compared with the high FE duodenal mitochondria (Ojano-Dirain et al., 2005a; Figure 3). The tissue variation in avANT and COX III mRNA expression that was observed in our studies could be explained by the observation that the cell response to oxidative stress varies depending on the degree of stress. For example, Li et al. (2002) observed upregulation and downregulation of intestinal mitochondrial genes with treatment of 0.4 or 4 mmol/L H2O2, respectively. Barzilai and Yamamoto (2004) also reported that 1) at very low levels of H2O2 (∼10 μM), proliferative cells show a significant mitogenic response, 2) at higher H2O2 (∼150 μM) cells undergo a temporary growth arrest, where energy is conserved by diminished expression of nonessential genes, and expression of shock and stress proteins is increased, and 3) at 250 to 400 μM of H2O2, cells enter a permanently growth-arrested state in which they appear to perform normal cell function but never divide again. Perhaps the constant turnover of cells that is well known to occur in intestinal epithelium may result in effective removal of oxidized proteins through sloughing of the epithelial cells, whereas in breast muscle, where there is very little cellular turnover, oxidized proteins accumulate. Thus, oxidized proteins are relatively lower in low FE duodenum compared with low FE breast muscle.

Figure 5. The mRNA expression of avian adenine nucleotide translocator (avANT), cytochrome oxidase III (COX III) and avian uncoupling protein (avUCP) in breast muscle obtained from broilers with high and low feed efficiency (FE). The mRNA values (in arbitrary units, AU) are expressed as ratio of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA values. Each bar represents the mean ± SEM for high and low (n = 7 to 8/group) FE birds. *P ≤ 0.05.

Mild uncoupling of oxidative phosphorylation, or increased proton conductance is suggested to attenuate mitochondrial ROS production (Skulachev, 1998; Echtay et al., 2002). Miwa et al. (2003) demonstrated that uncou-
plling agent (FCCP) reduced mitochondrial ROS production. Similarly, FCCP-induced uncoupling also lowered H$_2$O$_2$ production in low FE but not in high FE duodenal mitochondria (Ojano-Dirain et al., 2004b). Oxidative phosphorylation can be uncoupled by the actions of uncoupling proteins (UCP) located in the inner mitochondrial membrane where they mediate a regulated dissipation of the proton gradient generated by the respiratory chain (Ledesma et al., 2002). Thus, UCP provide an alternate route for proton translocation into the mitochondrial matrix other than through the ATP synthase, uncoupling respiration from ATP production (Vidal-Puig, 2000). The UCP-1, which is expressed only in brown adipose tissue, is the undisputed uncoupling protein and the only one that has an unchallenged thermogenic function (Nedergaard et al., 2005). In contrast, evidence for the thermogenic effect of the UCP-1 homologues, UCP-2 and UCP-3, remains weak to this point (Nedergaard et al., 2005). The UCP-2 and UCP-3 have been proposed to protect the cell against oxidative damage by inducing mild uncoupling, thus decreasing mitochondrial superoxide production (Brand et al., 2002, 2004).

An avian homologue of avUCP has also been identified in birds (Raimbault et al., 2001; Evock-Clover et al., 2002; Toyomizu et al., 2002) and may be involved in facultative thermogenesis (Dridi et al., 2004). The avUCP of Rhode Island Red chicken is 55, 70, and 71% homologous with mammalian UCP-1, UCP-2, and UCP-3, respectively (Raimbault et al., 2001). Avian UCP is predominantly expressed in skeletal muscle (Raimbault et al., 2001; Evock-Clover et al., 2002) with a much lower expression in kidney, liver, heart, lung, spleen, and adipose tissue (Evock-Clover et al., 2002). In the present study, we detected avUCP mRNA expression in breast muscle but not in the duodenal mucosa, which concurs with the findings of Dridi et al. (2004) on tissue distribution of avUCP. As illustrated in Figure 5, avUCP mRNA levels tended to be higher ($P = 0.068$) in breast muscle of low FE birds. Similarly, Raimbault et al. (2001) reported that leg muscle from a genetic line of chickens divergently selected for low FE (Bordas and Mérat, 1984) had higher avUCP mRNA expression than in birds from a high FE line, suggesting that avUCP may be involved in the control of body weight gain in birds through increased energy dissipation via mitochondrial oxidation (Dridi et al., 2004). In light of proposed mechanisms that uncoupling can lower oxygen radical production (Miwa et al., 2003), higher avUCP mRNA expression in breast muscle from low FE birds may be a mechanism to reduce the higher H$_2$O$_2$ production we have observed in low FE birds (Bottje et al., 2006). In fact, it has been reported recently that ROS production can be potentially downregulated by enhanced avUCP following fasting (Abe et al. 2006) and that acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, possibly via downregulation of avUCP (Mujahid et al., 2006).

Oxidative stress may also contribute to alterations in mitochondrial numbers within a tissue (Lee and Wei, 2006). Possibly via downregulation of avUCP (Mujahid et al., 2006) and that acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, which had higher FE compared with wild-type mice, had lower PGC-1$\alpha$ mRNA level. It has been also shown that exposure of chickens to a cold environment resulted in the prompt upregulation of avPGC-1$\alpha$ expression (Ueda et al., 2005). Some factors that are suggested to play a role in mitochondrial biogenesis include PPAR-$\gamma$, PGC-1$\alpha$, and nitric oxide (e.g., Wu et al., 1999; Nisoli et al., 2003). The nuclear transcription factor PPAR-$\gamma$ is activated by fatty acids and is the dominant regulator of the expression of genes encoding proteins that control adipocyte differentiation as well as fatty acid uptake and metabolism (Kliwer et al., 1997; Rosen et al., 1999). The PPAR-$\gamma$ is expressed in many cell types, such as epithelial, endothelial, and smooth muscle cells (Spiegelman, 1997). In chickens, PPAR-$\gamma$ plays a crucial role in fat deposition (Sato et al., 2004). As illustrated in Figure 6, PPAR-$\gamma$ mRNA expression was higher in the duodenum of high FE compared with the low FE group, but there were no differences in breast muscle PPAR-$\gamma$ mRNA expression (Figure 7). The PGC-1$\alpha$ is the most dominant regulatory protein in mitochondrial biogenesis (Wu et al., 1999). Mechanistically, PGC-1$\alpha$ stimulates nuclear respiratory factor-1 and mitochondrial transcription factor A expression, that in turn upregulate expression of nuclear and mitochondrial genes that encode mitochondrial proteins (Andersson and Scarpulla, 2001; Nisoli et al., 2003). Expression of avPGC-1$\alpha$ mRNA was not different between the high and low FE groups in the duodenum and breast muscle (Figure 6 and 7, respectively), although we have observed higher levels of PGC-1$\alpha$ protein expression in low FE muscle tissue (unpublished observations). The absence of significant differences in PGC-1$\alpha$ mRNA expression in the present study is in contrast with those of Nisoli et al. (2003) in which endothelial NOS$^{-/-}$ mice, which had higher FE compared with wild-type mice, had lower PGC-1$\alpha$ mRNA level. It has been also shown that exposure of chickens to a cold environment resulted in the prompt upregulation of avPGC-1$\alpha$ expression (Ueda et al., 2005).
whereas iNOS mRNA was relatively higher (different between the high and low FE birds (Figure 7), observed that iNOS expression in breast muscle was not...

Nitric oxide is synthesized from L-arginine by NO synthase (NOS) but this remains controversial (Brookes, 2004). Nitric oxide also mediates mucosal defense, influencing factors such as mucus secretion and mucosal blood flow (Pique et al., 1989). Nitric oxide is synthesized from L-arginine by NO synthase (NOS), which is present in 3 isoforms: neuronal, endothelial, and inducible. It is also proposed that mitochondria have a NOS isoform (mtNOS) but this remains controversial (Brookes, 2004). Unlike neuronal NOS and endothelial NOS, iNOS activity is independent of the calcium level in the cell; thus NO production by iNOS lasts much longer than from the other NOS isoforms and tends to produce much higher NO concentration (Reproductive and Cardiovascular Research Group, 2006). In the current study, we demonstrated a cGMP-dependent NO stimulation of mitochondrial biogenesis in brown adipocytes, in mouse white fat 3T3-L1 cells, and in human monocytic U937 cell lines. Nitric oxide is an important regulator of cellular function and is also a reactive nitrogen species that can act as a second messenger in signaling process (Barzilai and Yamamoto, 2004). Nitric oxide also mediates mucosal defense, influencing factors such as mucus secretion and mucosal blood flow (Pique et al., 1989).

As shown in Figures 4 to 6, the present study demonstrates that gene expression differs in the high and low FE birds and that gene expression varies between tissues. It appears that the increased H2O2 and higher protein oxidation, as we have observed in previous studies and in the current study, may cause differential expression of various genes in the high and low FE birds. Thus, in parallel with protein expression experiments, some genes, proteins, or both are potential candidates for the development of biomarker(s) for early detection of FE.

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