The Expression of Can and Camk is Associated with Lipogenesis in the Muscle of Chicken

ABSTRACT

Intramuscular fat (IMF) content in chickens significantly contributes to meat quality. The main objective of this study was to assess the expression of calcineurin (CaN) and Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK) in lipogenesis in chicken muscle. Chickens were slaughtered and sampled at 4, 8, and 16 weeks of age. IMF content and the expression of CaN subunits and CaMK isoforms were measured in the thigh muscle tissue. The results showed that the IMF contents were greater at 16 weeks compared with those at 4 and 8 weeks (p<0.05). Transcription of fatty acid synthase (FAS) and fatty acid translocase CD36 (FAT/CD36) mRNA significantly increased with age, from four to 16 weeks (p<0.05). The mRNA levels of CaN B and CaMK IV were significantly lower at 16 weeks than at four weeks (p<0.05), but CaMK II mRNA levels were significantly higher than at four weeks (p<0.05). In order to evaluate the role of CaMK and CaN in adipogenesis, SV cells were incubated in standard adipogenic medium for 24 h and treated with specific inhibitor of CaMK and CaN. The expressions of CCAAT/enhancer binding protein \(\beta\) (C/EBP\(\beta\)), sterol regulatory element-binding protein 1 (SREBP1), and peroxisome proliferation-activated receptor \(\gamma\) (PPAR\(\gamma\)) were dramatically enhanced by the CsA, CaN inhibitor (p<0.05). KN93, CaMK II inhibitor, dramatically repressed the expression of those lipogenic gene (p<0.05). These results indicated that CaN and CaMK had different effects on adipogenesis in the muscle of chickens.

INTRODUCTION

It is generally accepted that intramuscular fat (IMF) content is an important indicator of meat quality (Zhao et al., 2007; Choi & Kim, 2009; Hocquette et al., 2010). The problems of meat quality, such as sensory characteristics, tenderness and physical attributes, are related to fat accumulation in broilers. Adipogenesis is a well-regulated process regulated by many important transcription factors, such as CCAAT/enhancer binding protein \(\beta\) (C/EBP\(\beta\)), sterol regulatory element-binding protein 1 (SREBP1), and peroxisome proliferation-activated receptor \(\gamma\) (PPAR\(\gamma\)). PPAR\(\gamma\) is the main regulator of adipogenesis (Peter & Bruce, 2008). SREBP1 and C/EBP\(\beta\), expressed in the early stages of adipogenesis, induce the expression of PPAR\(\gamma\) at later stages of cell differentiation. Activation of PPAR\(\gamma\) plays a crucial role in the expression of adipocyte gene, such as fatty acid synthase (FAS) and fatty acid translocase CD36 (FAT/CD36) (Lin et al., 2011).

Adipogenesis is also regulated by calcium signaling pathways (Shi et al., 2000). The mechanism of increased Ca\(^{2+}\) levels repressing lipogenesis maybe contribute to the calmodulin (CaM) kinase cascade, which can activate both Ca\(^{2+}\)/CaM-dependent protein kinase (CaMK) and Ca\(^{2+}\)/calmodulin (CaM)-dependent phosphatase, calcineurin (CaN).
lipogenesis in the chicken skeletal muscle. The objective of the present study was to analyze the association between the expression of CaN and CaMK and lipogenesis in the chicken muscle. The expression of CaN and CaMK is associated with lipogenesis in the muscle of chicken.

**MATERIALS AND METHODS**

**Use of birds**

All animal procedures and care were performed in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China).

Ninety one-d-old female Bei Jing You (BJY) chickens (Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China) were raised starting from day 1. The starter feed (1–21 days) contained 20% crude protein and 12.01 MJ/kg, and the grower feed (>22 days) contained 19% crude protein and 12.55 MJ/kg. Feed and water were provided ad libitum during the experiment. Twelve birds were sacrificed per week at 4, 8, and 16 weeks of age, and the thigh muscles were collected. The right thigh muscles collected from the 12 birds sacrificed each week were stored at −20°C to determine intramuscular fat (IMF) content by ether extraction in a Soxhlet apparatus (Zhao et al., 2007), which was expressed as a the percentage of muscle weight (on dry matter basis). The left thigh muscles collected from the same birds were stored at −80°C for RNA extraction.

**Cell isolation and culture**

All reagents for cell culture were acquired from Sigma-Aldrich (St. Louis, MO), unless noted otherwise. The birds were sacrificed by CO₂ asphyxia. The pectoral muscle (PM) was isolated aseptically and finely minced after removing all visible connective tissue. The muscle stromal-vascular (SV) cells were obtained according to the procedure modified from a previous report (Hausman & Poulos, 2005).

Pectoral muscle tissue was digested for 30-40min by 0.1% collagenase type I (GIBCO, Grand Island, NY, USA) and then centrifuged at 1000 xg for 8min, after which the cell pellets were digested for 15-20min by 0.25% trypsin (GIBCO, Grand Island, NY, USA). The digesta were filtered through 200, 400 and 600 mesh screens to aseptically isolate the digested cells, which were subsequently centrifuged at 1000 xg for 5min.

Cells were rinsed with Dulbecco’s modified Eagle’s medium with F12 (DMEM/F12, 1:1,GIBCO,Grand Island, NY, USA), centrifuged at 1000 xg for 5 min, and re-suspended in 15mL growth media containing 84% DMEM/F12, 15% fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA), 1% HEPES, and penicillin 100U/mL, streptomycin 100U/mL. Cells were then plated on 6-well culture plates at 37°C in humidified (5%) CO₂ atmosphere. The cell cultures were aspirated from the plate 1 h after plating and fresh growth medium was added to each plate, as described by Hausman & Poulos (2005).

At 30% confluence, SV cells were incubated in adipogenic medium composed of 10% FBS /DMEM supplemented with insulin (10µg/mL), dexamethasone (1 µM), 3-isobutyl-1-methylxanthine (IBMX, 115ng/mL). In order to determine the role of CaMK and CaN on adipogenesis regulation, SV cells were cultured for 24 h in the presence of CaMK inhibitor KN93 (KN93, 5µmol/L) or CaN inhibitor cyclosporin A (CsA, 500ng/mL), respectively, or in the absence of these inhibitors as a control treatment (CON). The cells were collected at 24 h after initiating incubation for RNA extraction and mRNA analyses.

**Real-Time Quantitative PCR**

Total RNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer’s instructions. After DNase I (Promega, Beijing, China) treatment, total RNA concentrations were measured by spectrophotometry (optical density of 260 nm and 280 nm). All purified total RNA samples were diluted to 1 µg/µL in RNase-free water and stored at −80°C for quantitative reverse transcription-polymerase chain reaction (PCR) assays.

Reverse transcription of 2 µg RNA to first-strand cDNA was performed using a kit, according to the
manufacturer’s instruction (Promega, Beijing, China). Specific mRNAs were quantified by qPCR with an ABI 7500 Real-time Detection System (Applied Biosystems, USA) using a SYBR® Premix Ex Taq™ II kit (Takara, Dalian, China). The primers (Beijing Genome Institute, Beijing, China), based on chicken sequences, are listed in Table 1. Gene specific primers were designed by Primer Premier 5.0 from the corresponding chicken sequence to be intron spanning in order to avoid co-amplification of genomic DNA. β-actin was used as candidate housekeeping genes. The amplification was performed in a total volume of 20μL, containing 10 μL 2× SYBR Green I real-time PCR Master Mix(ABI), 1 μL forward primer (10 pmol), 1 μL reverse primer (10 pmol), 2 μL cDNA, 0.4 μL 50x ROX Reference Dye II, and 5.6 μL dH2O. The real-time PCR program started with denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Dissociation analysis of the amplification products was performed after each PCR to confirm that only one PCR product was amplified and detected.

Data were analyzed using the ABI 7500 SDS software (ABI), with the baseline set automatically by the software, and average dCT values (normalized using β-actin) were exported into Excel spreadsheets to calculate relative mRNA expression. The 2−ΔΔCt method of quantification was used to calculate the relative gene expression levels (Livak & Schmittgen, 2001).

### Statistical analysis

All data were submitted to the analysis of variance (ANOVA) procedure of SAS (version 8.0). Differences between the means were evaluated using Duncan’s multiple range test.

### RESULTS AND DISCUSSION

The accumulation of IMF and expression of the lipogenic gene in the thigh muscle

The IMF contents increased with broiler age, particularly from 8 to 16 weeks (p<0.05, Fig. 1), consistent with FAS, FAT/CD36 gene expression (Fig. 2). FAT/CD36 is a membrane receptor that facilitates long-chain fatty acid uptake. In avian species, the liver, and not the adipose tissue, is the main site of de novo FA synthesis. Therefore, the free fatty acid storage in the muscle mainly depends on membrane protein transportation systems, such as FAT/CD36 (Holloway et al., 2008). The metabolic activity of adipocytes inside the muscle tissue also effects lipid deposition in the muscle (Rollin et al., 2003), and therefore, many key lipogenic genes have been postulated as good markers for IMF content, such as FAS. The FAS and FAT/CD36 genes play essential roles in adipogenesis, and high muscle lipid content is accompanied by a greater abundance of the FAT/CD36 and FAS (Li et al., 2011), which is consistent with our results.

### Table 1 – Gene accession numbers and primer sequences

<table>
<thead>
<tr>
<th>Genes2</th>
<th>Primer sequence</th>
<th>Product, bp</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAT/CD36</td>
<td>F: 5'-TAATCATTGCACGATTCGCT-3' R: 5'-GCTTATTGGTTATATCTG-3'</td>
<td>104</td>
<td>DQ323177.1</td>
</tr>
<tr>
<td>FAS</td>
<td>F: 5'-CAATGGACCTTCATGCTCCCG-3' R: 5'-GCTGGTTACTGGAAGACAAC-3'</td>
<td>126</td>
<td>J04485</td>
</tr>
<tr>
<td>PPARγ</td>
<td>F: 5'-AGTCCTTCCCGCTGAGC-3' R: 5'-TCTCTGACTGCTCCTCACA-3'</td>
<td>168</td>
<td>AF470456.1</td>
</tr>
<tr>
<td>C/EBP β</td>
<td>F: 5'-GCCCGACTACACTACATCAGC-3' R: 5'-GTCCTCCTGAGCTTCCAGAT-3'</td>
<td>185</td>
<td>NM_205253</td>
</tr>
<tr>
<td>SREBP1</td>
<td>F: 5'-AAGGCAATGCTACATCGAGG-3' R: 5'-GGAGAACCCGGCAGCTTGA-3'</td>
<td>135</td>
<td>AJ414379.1</td>
</tr>
<tr>
<td>CaNα</td>
<td>F: 5'-TTCACCTTTCCTTTCTCCTC-3' R: 5'-AACACCATCCTTCTTCTTCCT-3'</td>
<td>151</td>
<td>AY324834.1</td>
</tr>
<tr>
<td>CaNβ</td>
<td>F: 5'-TGAGAGGCAACAGACAAAC-3' R: 5'-CAGGCAAGACCATAAGTGAGTAA-3'</td>
<td>245</td>
<td>NM_001030340.1</td>
</tr>
<tr>
<td>CAMK II</td>
<td>F: 5'-CAAAGGGGAAACAAAGCCG-3' R: 5'-CTTCAACATCTACTACGTTC-3'</td>
<td>385</td>
<td>AJ270104.1</td>
</tr>
<tr>
<td>CAMK IV</td>
<td>F: 5'-GCAGGGAGGAAAGAGCCG-3' R: 5'-GTGAAGAGCCGAAAGAGG-3'</td>
<td>105</td>
<td>NM_001034813</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5'-GCCGCTTCTTGAGTCACTA-3' R: 5'-CTCCTCTTCTTGAGTGA-3'</td>
<td>194</td>
<td>AF173612</td>
</tr>
</tbody>
</table>

1 All primers were designed from chicken sequences using the software Primer Premier 5.0

1 FAT/CD36 = fatty acid transporter Cd36; FAS = fatty acid synthase; PPARγ = peroxisome proliferator-activated receptor γ; C/EBP = CCAAT/enhancer binding protein; SREBP = sterol regulatory element-binding protein; CaN = calcineurin; CAMK = Ca²⁺/calmodulin-dependent protein kinase.
The expression of CaN and CaMK in the thigh muscle

The results obtained for mRNA levels of CaN subunits and CaMK isoforms in the thigh muscle are presented in Figure 3. The CaNA expression in the thigh muscle was not significantly influenced by bird age. CaNB and CaMK IV mRNA levels at 16 weeks were significantly lower than those at 4 weeks, but CaMK II mRNA levels were significantly higher at 16 weeks relative to 4 weeks (p<0.05).

In order to examine the role of CaMK and CaN in adipogenesis regulation, SV cells were cultured in an adipogenic medium and treated with KN93 and CsA, which is the specific inhibitor of CaMK II and CaN. The results showed that KN93 and CsA had opposite effects on the expression of lipogenic gene. Compared with the control, CsA (CaN inhibitor) dramatically increased the expression of C/EBPβ, SREBP1 and PPARγ; however, KN93 (CaMK II inhibitor) significantly repressed the expression of those lipogenic genes (p<0.05, Fig. 4).

CaN and CaMK are believed to be associated with muscle lipid metabolism (Long & Zierath, 2008). Adipogenesis is regulated by CaM kinase signaling pathways (Lin et al., 2011). The high level of intracellular Ca²⁺ in preadipocytes can inhibit adipogenesis, which may be due to the CaM kinase cascade. As downstream kinases, CaN and CaMK IV can be activated by CaM kinase. CaN and CaMK IV acts as a Ca²⁺-dependent molecular switch that negatively regulates the ability of 3T3-L1 cells to undergo adipocyte differentiation by preventing the expression of critical adipogenic transcription factors, such as C/EBPβ, SREBP1 and PPARγ (MacDonnell et al., 2009;
Lin et al., 2011). In present study, the role of CaMK II was different than those of CaMK IV and CaN in the expression of lipogenic gene. The SV cell, incubated with CsA and KN93, exhibited a different expression of lipogenic genes. CsA increased the expression of C/EBPβ, SREBP1 and PPARγ, whereas KN93 had opposite effects. Although as Ca2+-dependent enzymes CaN and CaMK II play a different role in lipogenesis, CaMK II appears to provide a Ca2+-independent pathway for stimulating adipogenesis (Meldolesi, 2008). CaMK II can inhibit CaN via the inhibition of NFAT nuclear translocation, which results in the activation of PPARγ and C/EBP α (MacDonnell et al., 2009). However, further research on the signal pathway of CaMK II in lipogenesis needs to be carried out.

CONCLUSION

Different expression of CaN and CaMK during the IMF deposition in chicken thigh muscle were demonstrated. The results of the present study indicate that transcription of CaN and CaMK was significantly correlated with lipogenesis in chicken thigh muscle.

ACKNOWLEDGEMENTS

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