



The Expression of *Ca_n* and *Ca_mk* is Associated with Lipogenesis in the Muscle of Chicken

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■ Keywords

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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 (*Ca_n*) and Ca²⁺/calmodulin-dependent protein kinase (*Ca_mk*) in lipogenesis in chicken muscle. Chickens were slaughtered and sampled at 4, 8, and 16 weeks of age. IMF content and the expression of *Ca_n* subunits and *Ca_mk* 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 *Ca_n* B and *Ca_mk* IV were significantly lower at 16 weeks than at four weeks ($p < 0.05$), but *Ca_mk* II mRNA levels were significantly higher than at four weeks ($p < 0.05$). In order to evaluate the role of *Ca_mk* and *Ca_n* in adipogenesis, SV cells were incubated in standard adipogenic medium for 24 h and treated with specific inhibitor of *Ca_mk* and *Ca_n*. The expressions of CCAAT/enhancer binding protein β (*C/EBP β*), sterol regulatory element-binding protein 1 (*SREBP1*), and peroxisome proliferation-activated receptor γ (*PPAR γ*) were dramatically enhanced by the CsA, *Ca_n* inhibitor ($p < 0.05$). KN93, *Ca_mk* II inhibitor, dramatically repressed the expression of those lipogenic gene ($p < 0.05$). These results indicated that *Ca_n* and *Ca_mk* 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 factors (*C/EBP α* , *C/EBP β*), sterol regulatory element-binding protein 1 (*SREBP1*), and peroxisome proliferator-activated receptors (*PPAR α* and *PPAR γ*). *PPAR γ* is the main regulator of adipogenesis (Peter & Bruce, 2008). *SREBP1* and *C/EBP β* , expressed in the early stages of adipogenesis, induce the expression of *PPAR γ* at later stages of cell differentiation. Activation of *PPAR γ* 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 Ca²⁺ levels repressing lipogenesis maybe contribute to the calmodulin (*Ca_m*) kinase cascade, which can activate both Ca²⁺/*Ca_m*-dependent protein kinase (*Ca_mk*) and Ca²⁺/calmodulin (*Ca_m*)-dependent phosphatase, calcineurin (*Ca_n*)



(MacDonnell *et al.*, 2009). Lin *et al.* (2011) showed that the Ca^{2+} /calmodulin-dependent protein kinase kinase 2 (*CaMKK2*), activated by the *CaM*, inhibits pre-adipocyte differentiation. *CaMK IV*, which is a downstream kinase, can be phosphorylated by *CaMKK2*. Many studies demonstrated *CaN* is involved in the regulation of adipocyte differentiation by preventing the expression of transcription factors of adipocyte differentiation (Neal & Clipstone, 2002). In addition, lipid mediators, such as prostaglandins 2 and IL-15, directly inhibit adipocyte differentiation via calcineurin-dependent mechanism that acts preventing the expression of the critical transcription factors *PPAR γ* and *C/EBP α* (Liu & Clipstone, 2007; Almendro *et al.*, 2009).

To date, *CaN* and *CaMK* functions in lipogenesis have been described by the adipocyte model. There is still little information on the role of *CaN* and *CaMK* in the lipogenesis in the chicken muscle. The objective of the present study was to analyze the association between the expression of *CaN* and *CaMK* and lipogenesis in the chicken skeletal muscle.

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-day-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 CO_2 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 $\times g$ 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 $\times g$ 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 $\times g$ 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%) CO_2 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 (10 $\mu\text{g}/\text{mL}$), 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, 5 $\mu\text{mol}/\text{L}$) 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 $\mu\text{g}/\mu\text{L}$ 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 20 μ L, containing 10 μ L 2 \times SYBR Green I real-time PCR Master Mix(ABI), 1 μ L forward primer (10 pmol), 1 μ L reverse primer (10 pmol), 2 μ L cDNA, 0.4 μ L 50 \times ROX Reference Dye II, and 5.6 μ L 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^{- $\Delta\Delta$ CT} 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 sequences¹

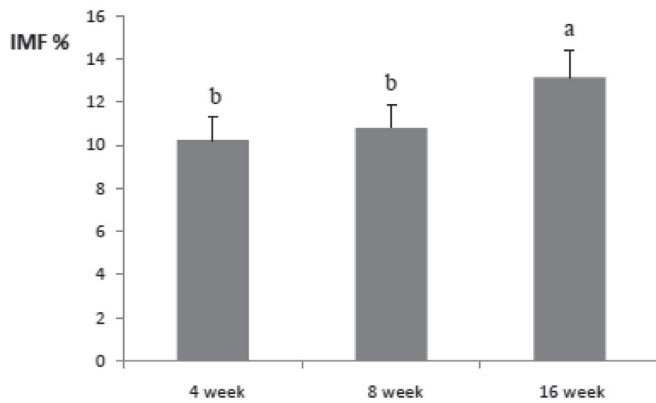
Genes ²	Primer sequence	Product, bp	Accession number
<i>FAT/CD36</i>	F: 5'-TAATCATCGCAGGTTCT-3' R: 5'-GCTTATTTGGGTTATTCACT-3'	104	DQ323177.1
<i>FAS</i>	F: 5'-CAATGGACTTCATGCCTCGGT-3' R: 5'-GCTGGGTAAGTGAAGCAACA-3'	126	J04485
<i>PPARγ</i>	F: 5'AGTCCTTCCCCTGACCAAA 3' R: 5'TCTCCTGCACTGCCTCCACA 3'	168	AF470456.1
<i>C/EBP β</i>	F:5' GCCCGACTACCTACATCAGC 3' R:5' GCTCCACTTTGGTCTCCACGAT 3'	185	NM_205253
<i>SREBP1</i>	F: 5' AAGGGCATTGACCTAAGCA 3' R: 5' GGAGAAGCCAGCCTGTGA 3'	135	AJ414379.1
<i>CaNα</i>	F: 5'-TTCAACTGCTCCCCTCAT-3' R: 5'-AACCATCTTCTCTGTCCCT-3'	151	AY324834.1
<i>CaNβ</i>	F: 5'-TGGAGGACGACAGACCC-3' R: 5'-CAGGCAAGACATAAGTGAGTAA-3'	245	NM_001030340.1
<i>CAMK II</i>	F: 5'-CCAAGGGGAACAACAGGC-3' R: 5'-CTTCAAGCAATCTACCGTCT-3'	385	AJ720104.1
<i>CAMK IV</i>	F: 5'-GCAGGCAGAAAGGGAC-3' R: 5'-GTGAAAGGCGAAGAAGG-3'	105	NM_001034813
<i>β-actin</i>	F: 5'-GCGGCTTTGGTGACTCTA-3' R: 5'-CTGCCCTCCTTGATGTG-3'	194	AF173612

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

²*FAT/CD36* = fatty acid transporter Cd36; *FAS* = fatty acid synthase; *PPAR γ* = 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.

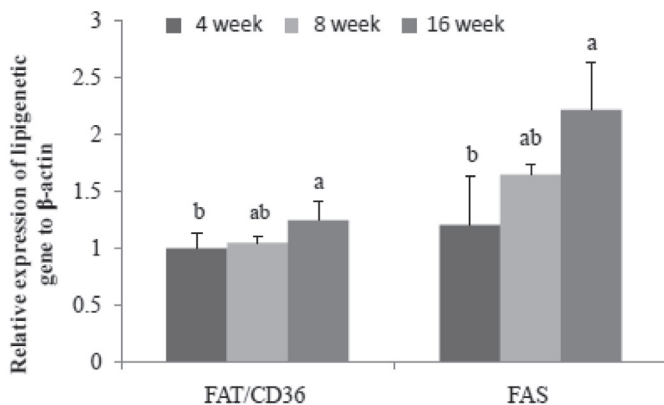


Figure 1 – The IMF deposition in the muscle of chickens at different ages.



(a,b) Different letters indicate significant differences among ages ($p < 0.05$). Values are shown as mean \pm standard deviation. IMF expressed as percentage based on thigh muscle tissue on dry matter basis.

Figure 2 – Relative mRNA expression levels of lipogenic genes in chickens of different ages.



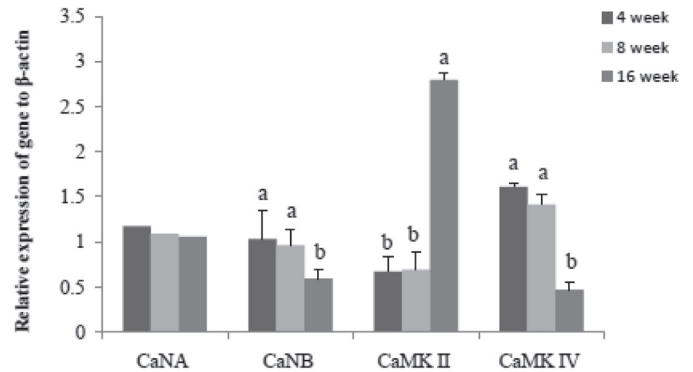
(a,b) Different letters indicate significant differences among ages ($p < 0.05$) for the same gene. Values are expressed as mean \pm standard deviation.

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

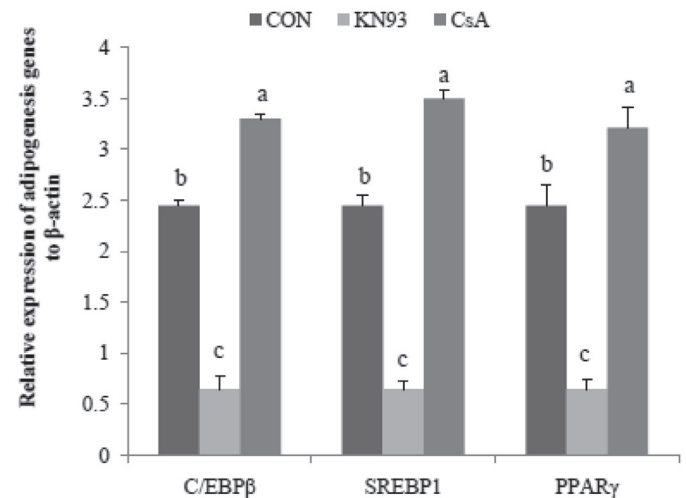
Figure 3 – Relative mRNA expression levels of *CaN* and *CaMK* subunits in chickens of different ages.



(a,b) Different letters indicate significant differences among ages ($p < 0.05$) for the same gene. Values are expressed as mean \pm standard deviation.

the expression of *C/EBP β* , *SREBP1* and *PPAR γ* ; however, KN93 (*CaMK II* inhibitor) significantly repressed the expression of those lipogenic genes ($p < 0.05$, Fig. 4).

Figure 4 – The effect of *CaN* and *CaMK* inhibitor on the adipogenic gene expression.



(a,b,c) Different letters indicate significant differences ($p < 0.05$) in the expression of a same gene. Values are expressed as mean \pm standard deviation.

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 Ca^{2+} 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 Ca^{2+} -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/EBP β/δ* , *SREBP1* and *PPAR γ* (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 Ca^{2+} -dependent enzymes *CaN* and *CaMK* II play a different role in lipogenesis, *CaMK* II appears to provide a Ca^{2+} -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 chickens thigh muscle.

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