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CaN and CaMK Expression Associated with Intramuscular Lipogenesis in Different Strain Chickens

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Abstract: The objective of this study was to assess the differential expression of CaN and CaMK during the improvement of lipogenesis in 2 strain chickens muscles. Twelve birds for each strain were slaughtered at 8 weeks for AA chickens and at 8, 16 weeks for BJY chickens. The results showed that the content of IMF and the expression of PPARγ, FAT/CD36 and FAS in muscle were significantly at 16 weeks higher than those for AA at 8 weeks and BJY at 8 weeks ($p<0.05$). Compared with AA chickens at 8 weeks, BJY at 16 weeks showed the significantly lower expression of CaN subunits and CaMK IV in muscle ($p<0.05$). But the expression of CaMK II was significantly higher at 16 weeks than 8 weeks in muscle ($p<0.05$). The expressions of C/EBPβ, SREBP1 and PPARγ were dramatically enhanced by CsA ($p<0.05$). KN93 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.

Key words: Chicken, calcineurin, Ca^{2+}/calmodulin-dependent protein kinase, intramuscular fat, effects

INTRODUCTION

In the last 50 years, the modern chickens grow very fast due to the improvement of genetics, nutrition, production and health. Compared with 50 years ago, the fast-chickens are marketed at about half the time and at twice the body weight (Barbut *et al*., 2008). But in Southern China and Hongkong markets, slow-growing chickens because of its meat quality are still highly popular with consumers (Zhao *et al*., 2012). The growth rate has great impact on the meat quality (Fanatico *et al*., 2007). The indigneous slow-growing chickens were judged to have better quality traits with regard to muscle fiber characteristics, cooking loss, drip loss, contents of IMF and IMP compared with meat of fast-growing chicken (Zhao *et al*., 2011; Sarsenbek *et al*., 2013). Especially Intramuscular Fat (IMF) content is important indicators of meat quality, influencing the flavor, texture and visual appeal of the meat (Zhao *et al*., 2007; Choi and Kim, 2009; Eggert *et al*., 2002). The high meat quality of slow-growing chicken may attribute to the genetic selection for high IMF content (Zhao *et al*., 2007; Chen *et al*., 2008).

Adipogenesis is a well-regulated process regulated by many important transcription factors such as CCAAT/Enhancer-Binding Protein factors (C/EBPα, C/EBPβ), Sterol Regulatory Element-Binding Protein 1 (SREBP 1) and Peroxisome Proliferator-Activated Receptors (PPARα and PPARγ). PPARγ is the master regulator of adipogenesis (Tontonoz and Spiegelman, 2008). SREBP1 and C/EBPβ, expressed in the early stages of adipogenesis, induce the expression of PPARγ at later stages of cell differentiation. Activation of PPARγ plays an crucial roles in the expression of adipocyte gene including Fatty Acid Translocase CD36 (FAT/CD36), Fatty Acid Synthase (FAS) and others.

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) (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 preadipocyte differentiation. CaMK IV is a downstream kinase can be phosphorylated by CaMKK2. Many studied demonstrated CaN is involved in the regulation of adipocyte differentiation by preventing the expression of transcription factors of adipocyte differentiation (Neal and Clipstone, 2002). In addition, lipid mediators such as Prostaglandins 2, IL-15 directly inhibits

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adipocyte differentiation via calcineurin-dependent mechanism that acts to prevent the expression of the critical transcription factors PPARγ and C/EBPα (Liu and Clipstone, 2007; Almendro *et al*., 2009).

Up to now, functions of CaN and CaMK in lipogenesis come from the adipocyte model. Researchers still have a little information about the CaN and CaMK in the role of lipogenesis in the muslce of fast-growing and slow-growing chickens. The objective of the present study was to analyze the differential expression of CaN and CaMK during the accumulation of lipogenesis in the different chicken genotype.

MATERIALS AND METHODS

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).

Animals and Sample collection: Ninety each of female 1, day slow-growing genotype Bei-Jing-You (BJY) and fast-growing genotype AA birds (the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China) were raised starting from 1 day of age. All birds were randomly distributed into 6 replicate groups for each breed; each group is comprised of 15 BJY birds and 15 AA birds, respectivly. The starter ration (1-21 days) with 200 g crude protein/kg diet and 12.01 MJ kg^{-1} differed only slightly from that used in the grower (>22 day) phase, 190 g crude protein/kg diet and 12.55 MJ kg^{-1}. Feed and water were provided *ad libitum* during the experiment.

Birds were slaughtered at market ages: AA chickens at 8 weeks age (AA 8 weeks) and BJY chickens at 16 weeks age (BJY 16 weeks). In order to compare the lipid metabolism with same age of AA chickens, BJY chickens were also slaughtered at 8 weeks age (BJY 8 weeks). Following a 12 h overnight fast, 2 birds of similar weight from each replicate were killed by exsanguination. Removing all visible connective tissue and adipose, the breast muscle and thigh muscle were collected. The right breast and thigh muscles were stored at -20°C for Intramuscular Fat (IMF) determination by Soxhlet extraction as described by Zerehdaran *et al*. (2004) and Zhao *et al*. (2007). The left breast abd thigh muscles were stored at -80°C for RNA extraction.

Cell isolation and culture: All chemicals for cell culture were bought from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. The 4~7 days old BJY chicken was killed and Pectrail Muscle (PM) was isolated aseptically and finely minced after removing all visible connective tissues. The muscle Stromal-Vascular (SV) cells was obtained as the procedure modified from previous report (Hausman and Poulos, 2005).

Tissues of PM were digested 30~40 min by 0.1% collagenase type I (GIBCO, Grand Island, NY, USA) and then digesta were centrifuged at 1000 r min^{-1} for 8 min. Then, the cell pellets were digested 15~20 min by 0.25% trypsin (GIBCO, Grand Island, NY, USA). The digesta were passed through 200, 400 and 600 screen mesh filter to isolate aseptically and digested cells and centrifuged at 1000 r min^{-1} for 5 min. Cells were rinsed with Dulbeco's modified Eagle's medium with F12 (DMEM/F12, 1:1, GIBCO, Grand Island, NY, USA), centrifuged at 1000×g for 5 min and re-suspended in 15 mL growth media containing 84% DMEM/F12, 15% Fetal Bovine Serum (FBS, GIBCO, Grand Island, NY, USA), 1% HEPES and penicillin 100 U mL^{-1}, streptomycin 100 U mL^{-1}, plated in 6 wells plate 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 were added to each plate as described by Hausman and Poulos (2005).

At 30% confluence, SV cells were incubated in a adipogenic medium composed of 10% FBS/DMEM supplemented with insulin (10 μg mL^{-1}), dexamethasone (1 μM), 3-Isobutyl-1-Methylxanthine (IBMX, 115 ng mL^{-1}) for 24 h. To examine the role of CAMK and CaN on adipogenesis regulation, the SV cells were cultured, respectively for 24 h in the presence of CaMK inhibitor KN93 (KN93, 5 umol L^{-1}) or CaN inhibitor cyclosporin A (CsA, 500 ng mL^{-1}) or absence of these inhibitors as Control (CON). The cells were collected at 24 h after initiating incubation for RNA extracting and mRNA analyses.

Total RNA preparation and quantitative RT-PCR (qPCR): Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. All purified RNA samples were diluted in RNase-free water to 1 μg μL^{-1} concentration and stored at -80°C for qRT-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

Table 1: Gene accession numbers and primer sequences[1]

Genes[2]	Primer sequence	Product (bp)	Accession number
FAT/CD36	F: 5'-TAATCATCGCAGGTTCT-3'	104	DQ323177.1
	R: 5'-GCTTATTTGGGTTATTCAGT-3'		
FAS	F: 5'-CAATGGACTTCATGCCTCGGT-3'	126	J04485
	R: 5'-GCTGGGTACTGGAAGACAAACA-3'		
PPARγ	F: 5'AGTCCTTCCCGCTGACCAAA 3'	168	AF470456.1
	R: 5'TCTCCTGCACTGCCTCCACA 3'		
C/EBPβ	F: 5' GCCCGACTACACCTACATCAGC 3'	185	NM_205253
	R: 5' GCTCCACTTTGGTCTCCACGAT 3'		
SREBP1	F: 5' AAGGGCATTGACCTAAGCA 3'	135	AJ414379.1
	R: 5' GGAGAAGCCAGCCTGTGA 3'		
CaNα	F: 5'-TTCAACTGCTCCCCTCAT-3'	151	AY324834.1
	R: 5'-AACCATCTTCTTCTGTCCCT-3'		
CaNβ	F: 5'-TGGAGGACGACAGACCC-3'	245	NM_001030340.1
	R: 5'-CAGGCAAGACATAAGTGAGTAA-3'		
CAMK II	F: 5'-CCAAGGGGAACAACAGGC-3'	385	AJ720104.1
	R: 5'-CTTCAAGCAATCTACCGTCT-3'		
CAMK IV	F: 5'-GCAGGCAGAAAGGGAC-3'	105	NM_001034813
	R: 5'-GTGAAAGGCGAAGAAGG-3'		
β-actin	F: 5'-GCGGCTTTGGTGACTCTA-3'	194	AF173612
	R: 5'-CTGCCTTCCTTGGATGTG-3'		

[1]All primers were designed from chicken sequences using the software Primer Premier 5.0; [2]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 = Ca^{2+}/Calmodulin-dependent protein Kinase

DNA. The amplification was performed in a total volume of 20 μL, 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 50×ROX Reference Dye II and 5.6 μL dH_2O. The real-time PCR program started with denaturing at 95°C for 1 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Dissociation analysis of amplification products was performed after each PCR to confirm that only one PCR product was amplified and detected.

Data were analyzed with ABI 7500 SDS Software (ABI) with the baseline being set automatically by the software and values of average dCT (normalized using β-actin) was exported into excel for the calculation of relative mRNA expression. The $2^{-\Delta\Delta C_T}$ Method of quantification (Livak and Schmittgen, 2001) was used to calculate the relative expression levels of each gene.

Statistical analysis: All data were analyzed by ANOVA using SAS Software (Version 8.0). Differences between the means were assessed using Duncan's multiple range test. The $p<0.05$ was considered significant.

RESULTS

The contents of breast and thigh IMF are shown in Fig. 1. There was an insignificant difference in breast IMF content between AA chickens and BJY chickens at 8 weeks age. But the IMF content of breast was higher at 16 weeks age than 8 week age ($p<0.05$). The thigh IMF contents were significantly higher in BJY chickens than in AA chickens moreover, IMF content of BJY chickenss were higher at 16 weeks than at 8 weeks ($p<0.05$).

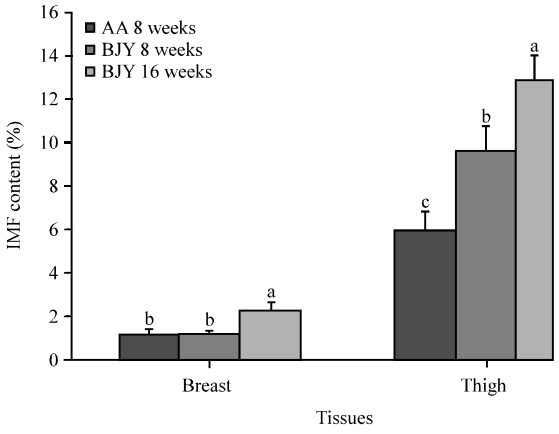

Fig. 1: IMF content of AA and BJY chicken at different age. [a-c]Means within same tissues with different superscripts differ significantly ($p<0.05$). Data are expressed as mean±SD (n = 12); IMF expressed as percentages on the basis of weight of dry muscle tissue

The metabolic activities of adipocytes inside the muscle tissue also effects lipid deposition in the muscle. The expression of lipogenic genes in muscle was shown in the Fig. 2. In both thigh muscle and breast muscel, the expressions of PPARγ, FAT/CD36 and FAS were slightly but not significantly, higher in 8 weeks BJY chickens than in 8 weeks AA chickens. The expression of PPARγ, FAT/CD36 and FAS were significantly higher in 16 weeks BJY chickens than in 8 weeks both AA and BJY chickens ($p<0.05$).

Results on the mRNA levels of CaN and CaMK in the thigh and breast muscle are presented in Fig. 3. Compared

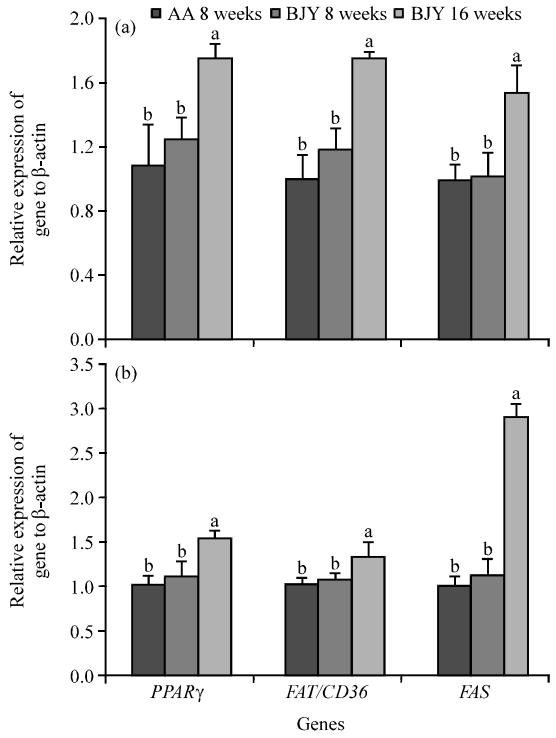

Fig. 2: Expression profiles of lipogenic genes in; a) thigh and b) breast muscle. [a-c]Means within same genes with different superscripts differ significantly ($p<0.05$). Data are expressed as mean±SD(n = 12)

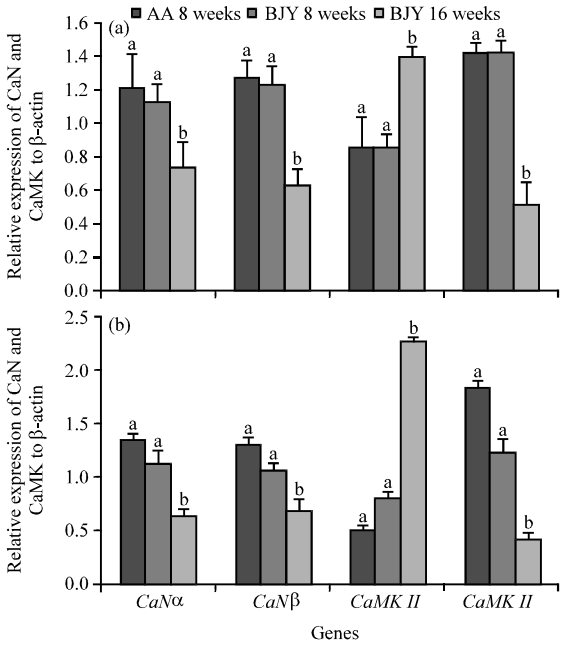

Fig. 3: Expression profile of CaN and CaMK in; a) thigh and b) breast muscle. [a-c]Means within same genes with different superscripts differ significantly ($p<0.05$). Data are expressed as mean±SD (n = 12)

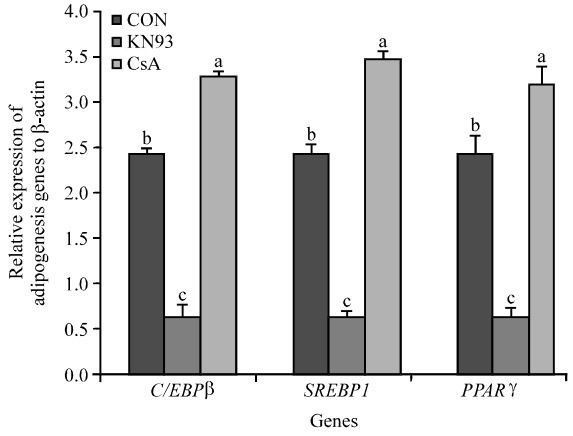

Fig. 4: The effect of CaN and CaMK inhibitor on the adipogenic gene expression. [a-c]Different letters indicate significant differences ($p<0.05$) at the same gene. Values are means±standard deviations

with the 8 weeks AA chickens, the 16 weeks BJY showed the significantly lower expression of CaN subunits and CaMK IV in both breast and thigh muscle ($p<0.05$) but the expression of CaMK II was significantly higher at 16 weeks than 8 weeks in both breast and thigh muscle ($p<0.05$). In BJY chickens there was a significant difference expression of CaN and CaMK in breast and thigh muscle between 8 and 16 weeks chickens.

To examine the role of CaMK and CaN on adipogenesis regulation, the SV cells were cultured in an adipogenic medium and treated with KN93 and CsA, the specific inhibitor of CaMK II and CaN. The results showed that KN93 and CsA had opposite effect on the expression of lipogenic gene. Compared with control. CsA, CaN inhibitor, dramatically promoted the expression of C/EBPβ, SREBP1 and PPARγ. But KN93, CaMK II inhibitor dramatically repressed the expression of those lipogenic gene ($p<0.05$, Fig. 4).

DISCUSSION

Intramuscular Fat (IMF) content plays a key role in various quality traits of meat. The quantity of fat deposited increases faster and earlier in the fast-growing chickens than in slow-growing chickens (D'Andre *et al*., 2013). The breast lipid contents of fast-growing chickens significantly increases with age increasing from 35-49 days, maximum level at 56 days (Baeza *et al*., 2012). But for slow-growing chickens, the accumulation of IMF in breast and thigh increases after 56 days. So, researchers compared the IMF content of AA chicnekns and BJY chickens at marketed age.

The IMF content of avian depends not only on the synthesis in the liver but also on the muscle growth rate. Animals having a high muscularity with a high glycolytic activity display a reduced development of IMF (Hocquette *et al*., 2010). Therefore, the fast-growing chickens with high muscularity have lower IMF contents compared with slow-growing chicken. The present results were also consistent with the IMF content of other research (Sarsenbek *et al*., 2013). But when slow-growing chickens were fed with free-range or outdoor, the IMF contents was lower than those for fast-growing chickens (Fanatico *et al*., 2007; Sirri *et al*., 2011). So, the IMF content may be also associate with feeding strategy and production system.

In avian, the liver but not adipose tissues is the main site of de novo fatty acid synthesis. So, the free fatty acid storage in the muslce mainly depends on the several membrane protein trasportion system such as FAT/CD36 (Holloway *et al*., 2008). Additional studies have shown that slow-growing chickens exhibited higher amounts of total polyunsaturated fatty acids compared with fast-growing chickens (Dal Bosco *et al*., 2012; Wang *et al*., 2013). In present study, slow-growing chickens exhibites the high expression of FAT/CD36 which was consistent with the higher content of IMF in the slow-growing chickens.

Meanwhile, the lipid synthesis in the muscle is negligible (Rollin *et al*., 2003). Therefore, many key lipogenic genes have been postulated as good markers for IMF content such as FAS, PPARγ. FAS is a key multifunctional enzyme contributing to the synthesis of fatty acids. PPARγ participates in a pathway of fat cell development and in inducing an array of genes related to lipid metabolism (Lin *et al*., 2011). The varities of IMF content may attribute to different profile of gene expression in the AA and BJY chickens.

CaN and CaMK are believed to be associated with muscle lipid metabolism (Long and 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 down stream 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 PPARγ (MacDonnell *et al*., 2009; Lin *et al*., 2011). In present study, the role of CaMK II differed with CaMK IV and CaN in the expression of lipogenic gene. In order to clarify the role of CaN and CaMK in the lipogenesis, the SV cells were administrated with CsA and KN93. The results showed that CaN and CaMK exhibited the different expression of lipogenic gene. CsA increased the expression of C/EBPβ, SREBP1 and PPARγ. But KN93 had opposite effects on the those adipogenic gene. Although, as Ca^{2+}-dependent enzymes, CaN and CaMK II play the different effects on the lipogenesis. CaMKII appears to provide a Ca^{2+}-independent pathway for stimulating adipogenesis (Meldolesi, 2008). CaMK II can inhibit the CaN via the inhition of NFAT nuclear translocation which results in the activation of PPARγ and C/EBPα (MacDonnell *et al*., 2009). But researchers still carry out further research to study the signal pathway of CaMK II in lipogenesis.

CONCLUSION

At the market age, BJY chickens had a significant higher IMF content and lipogenic gene expression than AA chickens. But in contrast, the expression of CaN and CaMK IV in AA chicken were higher than those of BJY chicken. The results of SV cell treated with specific inhibitor showed that the CaN and CaMK is invovled in the lipogenesis in the muscle of Chickens.

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