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Effects of conjugated linoleic acid on the expression levels of miR-27 and miR-143 in pig adipose tissue

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ABSTRACT. In this study, we evaluated the effect and possible mechanism of action of dietary conjugated linoleic acid (CLA) on pig body fat deposition. Landrace piglets (N = 48) were randomly divided into three groups, which were fed diets containing 0% (control), 1%, or 2% CLA. Dorsal and abdominal subcutaneous adipose tissues were collected, and real-time polymerase chain reaction (PCR) was used to determine the expression of adipocyte differentiation marker genes and associated microRNAs (miRNAs). Our results indicated that dietary CLA significantly decreased body fat deposition in the pig dorsum. The expression of adipocyte differentiation marker genes, including peroxisome proliferator-activated receptor (PPAR)-γ and CCAAT/enhancer-binding protein α (C/EBPα) were not affected, whereas the expression of fatty acid binding protein 4 (FABP4) was significantly enhanced ($P < 0.05$). The expression of miR-27 and miR-143 in adipose tissue was significantly decreased. Data analysis indicated a significant negative

correlation between miR-27 and FABP4 expression in the dorsal subcutaneous adipose tissue. In addition, the expression of miR-143 and miR-27 exhibited a significant negative relationship with FABP4 and PPARγ in the abdominal subcutaneous adipose tissue. Thus, miRNA levels in adipose tissues could be modulated by CLA, thereby affecting adipose metabolism.

Key words: Conjugated linoleic acid; MicroRNA; Adipose tissue; Pig; Gene expression

INTRODUCTION

MicroRNAs (miRNAs), which are 18 to 25 nucleotides in length, are a class of non-coding RNAs present in plants and animals. Substantial evidence indicates that more than 60% of plant and animal mRNA is subject to miRNA regulation. miRNAs mediate the degradation or inhibit the translation of target mRNAs by forming RNA-induced silencing complexes through complete or partial pairing of seed regions with target mRNA sequences (Zeng et al., 2003; Gregory et al., 2004; Friedman et al., 2009). Several miRNAs in adipose tissues have been identified, but only a few of these exhibit specific regulatory functions and definite mRNA targets. To date, miR-27 and miR-143 have been a focus of studies on miRNAs because both have vital functions in adipose metabolism and the development of adipocytes in animals through targeting peroxisome proliferator-activated receptor (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), extracellular signal-related kinase 5 (ERK5), and fatty acid binding protein 4 (FABP4) (Chen et al., 2004; Esau et al., 2004; Kajimoto et al., 2006; Xie et al., 2009; Zhang et al., 2010).

Conjugated linoleic acid (CLA) is an octadecadienoic acid containing a conjugated double bond that performs several physiological functions. Studies have indicated that CLA can enhance immunity, prevent tumor formation, inhibit oxidative stress, promote muscle growth, and reduce body fat deposition (MacDonald et al., 2000; Wang et al., 2004a; De La Torre et al., 2005). Through the reduction of body fat deposition, CLA is thought to have an anti-obesity activity. Although the mechanism by which CLA reduces fat deposition remains unclear, the effect of CLA on fat deposition reduction most likely occurs via inhibition of adipocyte proliferation and differentiation, thereby promoting the hydrolysis of intracellular triglycerides and inducing adipocyte apoptosis (Evans et al., 2000; Wang et al., 2004b).

This study aimed to investigate the effects of dietary CLA on miR-27 and miR-143 expressions and identify their target genes in pig adipose tissue to determine whether the anti-obesity function of CLA is associated with miRNA changes. The results could provide experimental evidence for future studies on obesity and metabolic syndrome.

MATERIAL AND METHODS

Animals and diets

The present study was approved by the Ethics Committee of Chongqing Academy of Animal Science (Approval No. 2012-27), and the animal euthanasia and sample collection were in strict accordance with the requirements of the Ethics Procedures and Guidelines of

China.

Healthy landrace piglets were purchased from a commercial farm and fed in a standard experimental piggery of the Chongqing academy of animal science. Forty-eight piglets were randomly selected and divided into three groups (N = 16 per group). The control group was fed a corn-soybean meal basal diet, which was formulated according to the nutrient requirements for growing swine (National Research Council, USA, 1998) with body weights ranging from 10 kg to 20 kg and from 20 kg to 50 kg. The trial groups were fed a diet of 1% (group 1) or 2% (group 2) CLA (purity 61.2%; AuHai Biotech Co. Ltd., Qingdao, China). CLA was used as a substitute for soybean oil in the basal diet. The experiment lasted 30 days.

Food intake and body weight gain

Daily feed consumption was determined during the experiment. The body weight of piglets was measured on days 1, 15, and 30 of the experimental period, and the average daily gain and feed conversion ratio were calculated.

Body fat deposition

Five randomly selected piglets from each group were euthanized on day 30. Dorsal and abdominal subcutaneous adipose tissues were isolated and weighed. The thickness of the subcutaneous adipose tissue in the neck was measured using a vernier caliper.

RNA extraction and gene expression analysis

Total RNA from fat tissue was extracted using RNAiso Plus (TAKARA, Otsu, Shiga, Japan) according to manufacturer instructions. Isolated RNA was then quantified using a BioPhotometer Plus spectrophotometer (Eppendorf, Hamburg, Germany), and the integrity of the isolated RNA was confirmed by 1.5% agarose gel electrophoresis. Gene expression was assessed by real-time polymerase chain reaction (PCR) performed using a StepOne system (ABI, Foster City, CA, USA). For miRNA measurements, RNA was initially reverse transcribed and amplified with gene-specific primers by using a SYBR® PrimeScript™ miRNA RT-PCR kit (TAKARA). The kit included a reverse transcription primer. To evaluate the adipocyte genes of interest, we reverse transcribed the total RNA to cDNA by using a PrimeScript™ RT reagent kit and amplified the total RNA with gene-specific primers by using the SYBR Premix Ex Taq™ II (TAKARA). Table 1 lists the primer sequences. A target gene was relatively quantified based on efficiency and the crossing point deviation of an unknown sample versus a control sample, and then expressed by comparing with a reference gene used to normalize cDNA. U6 small nuclear RNA and β-actin were used as internal genes for miRNA and mRNA expression analyses, respectively.

Statistical analysis

The results are reported as means ± SE and statistically compared with the control group or within the groups using one-way ANOVA and Tukey's multiple comparison tests. Linear relationships between the key variables were determined using Pearson's correlation coefficients. SPSS for Windows version 13 (SPSS, Chicago, IL, USA) was used for the analyses.

Table 1. Primer sequences.

Genes	Primer sequences (5'→3')
miR-27	TTCACGGTGGCTAAGTTCTGC
miR-143	TGCGATGAACCACTGAACCTC
PPARγ	F: ATGAAGAGGAACCACATTA
	R: TTATTGCCTCAGTAGCTTG
CEBPα	F: AGAAGATCAACTGAGTGAACT
	R: AGAGCTGAGAAACTAACGTG
FABP4	F: GCTACGACGGCACCTATTACAG
	R: CACGATGCTGGACAGACAGT
U6	F: TTATGGGTCCTAGCCTGAC
	R: CACTATTGCGGGTCTGC
β-actin	F: GCGGCATCCACGAAACTAC
	R: TGATCTCCTTCTGCATCCTGTC

RESULTS

Body fat deposition in piglets

Our results clearly showed that CLA supplementation significantly affects body fat deposition in piglets (Table 2). In pigs that were fed CLA for 30 days, both body weight and feed intake decreased slightly ($P > 0.05$). However, the body fat weight and skin fat thickness of CLA-fed pigs were significantly reduced, particularly the back fat ($P < 0.05$). In addition, the decrease in body fat was related to the CLA supplementation dose.

Table 2. Body weight and body fat deposition of pigs.

Items	Control	1%CLA	2%CLA
Initial body weight (kg)	13.91 ± 1.68	13.91 ± 1.55	13.91 ± 1.37
Final body weight (kg)	30.39 ± 1.36	28.10 ± 2.38	28.46 ± 1.47
Feed intake (kg)	36.13 ± 2.77	30.96 ± 3.51	31.43 ± 2.27
F/G	2.19 ± 0.05	2.18 ± 0.03	2.16 ± 0.4
Back fat weight (g)	370.48 ± 33.63^{A}	319.60 ± 17.64^{B}	297.68 ± 14.32^{B}
Abdominal fat weight (g)	179.04 ± 27.70	172.24 ± 19.69	166.48 ± 9.35
Subcutaneous fat thickness (neck) (cm)	2.43 ± 0.21	2.24 ± 0.17	2.17 ± 0.18

Different superscripts discriminate differences ($P < 0.05$) between groups.

miRNA expression of adipose tissue

qPCR results showed that the expression levels of miR-27 and miR-143, which are important miRNAs in the white fat tissue, were significantly decreased ($P < 0.05$) in the back fat and abdominal fat tissues of CLA-fed pigs as compared with the control pigs (Figure 1). The 2% CLA-fed pigs exhibited minimum expression levels of miR-27 and miR-143. These results also revealed that nutrients could affect the miRNA expression levels *in vivo*.

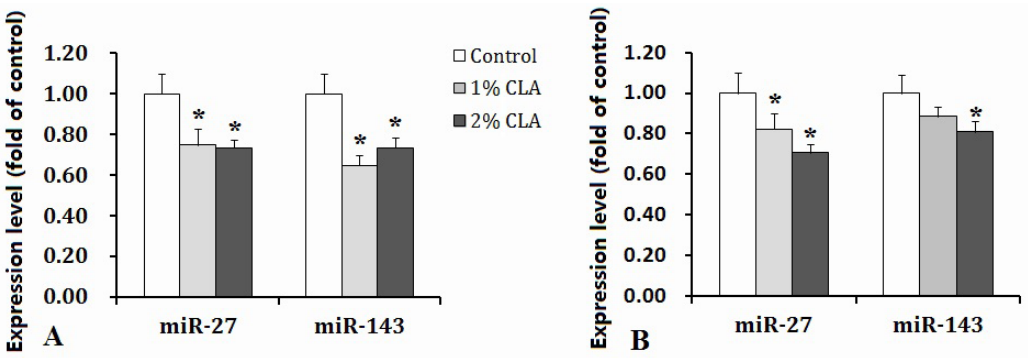

Figure 1. Conjugated linoleic acid (CLA) supplementation reduced the expression levels of miR-27 and miR-143 in back fat (**A**) and abdominal fat (**B**) of growing pigs. *P < 0.05 as compared with control.

Expression of adipocyte differentiation marker genes

PPARγ and C/EBPα, the two key transcription factors, are considered as adipocyte differentiation markers. The expression levels of these proteins increase in adipose tissues during adipocyte differentiation. As shown in Figure 2, no obvious differences were observed in PPARγ and C/EBPα expressions among the three pig groups (P > 0.05). FABP4 promotes adipocyte uptake and free fatty acid discharge, particularly long-chain fatty acids. High FABP4 expression may be a marker of adipocyte differentiation. Our data showed that the two CLA-fed pig groups exhibited higher FABP4 expression in the back fat tissue (P < 0.05) and that abdominal fat expression of FABP4 was increased in the 2% CLA-fed pigs (P < 0.05).

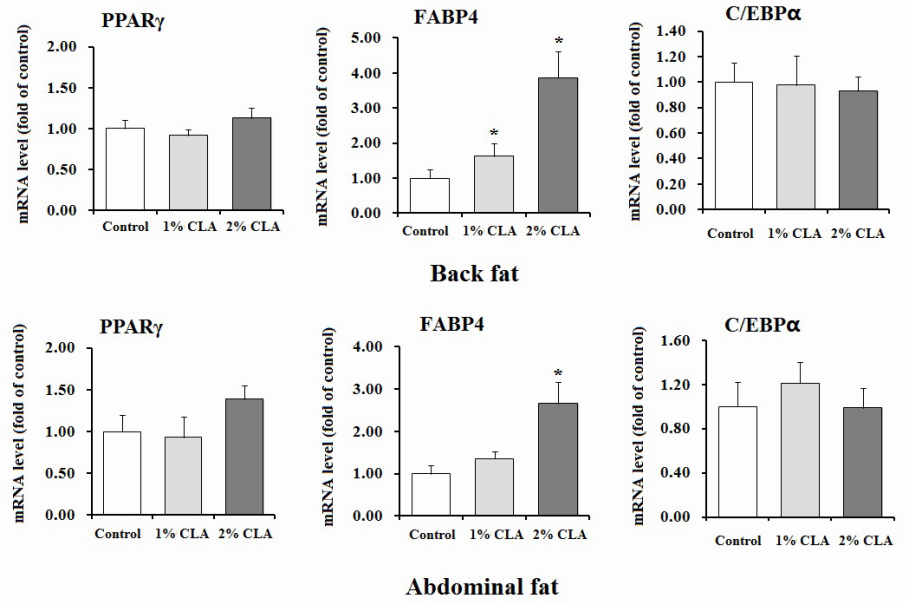

Figure 2. Expression level of adipocyte differentiation marker genes in back fat (upper) and abdominal fat (lower) of growing pigs. *P < 0.05 compared with control.

Correlation between miRNA expression and adipocyte differentiation marker genes

We further analyzed the expression of adipocyte-associated genes to determine the potential relationship between the expression of selected miRNAs and the adipocyte phenotype (Table 3). In the back fat tissue, miR-27 expression significantly correlated with FABP4 expression (-0.722, $P < 0.05$). In the abdominal fat tissue, miR-27 and miR-143 expressions were significantly correlated with PPARγ (miR-27, -0.741, $P < 0.05$; miR-143, -0.682, $P < 0.05$) and FABP4 expressions (miR-27, -0.918, $P < 0.01$; miR-143, -0.698, $P < 0.05$). No significant correlation was observed between the selected miRNAs and C/EBPα.

Table 3. Correlations between miRNA and adipocyte differentiation genes in piglets with conjugated linoleic acid (CLA).

	miR-27	miR-143
Back fat		
PPARγ	-0.323	-0.230
C/EBPα	0.550	0.535
FABP4	-0.722*	-0.477
Abdominal fat		
PPARγ	-0.741*	-0.682*
C/EBPα	-0.47	-0.187
FABP4	-0.918**	-0.698*

Comparison between miRNAs and adipocyte differentiation marker genes expression was done by the Pearson Chi-square test. Statistical significance (2-tailed). *$P < 0.05$, **$P < 0.01$.

DISCUSSION

Among animals, pigs exhibit the highest fat content and similar physiological characteristics as humans. As such, pigs are an ideal animal model to study obesity and relative syndromes. Furthermore, the livestock industry in China has focused on several approaches to decrease body fat deposition, increase dressing percentage, and improve pork quality because of the high consumption in Chinese agricultural commodity markets.

Adipocyte proliferation, differentiation, metabolism, and death in animals are regulated in a programmed manner by crosstalk between numerous regulatory factors. These processes and factors influence body fat deposition. Fat deposition in pigs occurs sequentially in the following parts: subcutaneous, visceral, and intramuscular adipose tissues (Anderson and Kauffman, 1973). The metabolic patterns and growth rates in different adipose tissues are distinct. For example, visceral and subcutaneous adipose tissues vary in terms of secretion, lipogenesis, and adipose differentiation. In this study, the dorsal and abdominal subcutaneous adipose tissues of pigs were collected for experiments, and interestingly, our data differed between the tissues. Dietary CLA significantly reduced back fat deposition, thereby exhibiting a decrease in the abdominal fat deposition. The differences in these results may be attributed to sequential adipose deposition. Moreover, these findings indicated that dietary CLA is effective in mature adipocytes. In addition, CLA supplementation remarkably downregulated the expression levels of miR-27 and miR-143 in the adipose tissues of piglets, with a strong effect observed with 2% CLA .These results also indicated that miRNA expression levels in animals could be regulated by dietary nutrients.

PPARγ, C/EBPα, and FABP4 are well-known, essential regulatory factors in fat metabolism. They are considered as marker genes of metaphase and anaphase during adipocyte

differentiation. Both PPARγ and C/EBPα are vital transcription factors that influence the expression or function of target genes by binding to the transcription binding sites of target genes (Miller et al., 2003; König et al., 2009). Some reports speculated that PPARγ and C/EBPα are the targets of miR-27 and miR-143 (Lin et al., 2009; Chen et al., 2014). However, our results showed that PPARγ and C/EBPα expressions were not significantly altered as compared to the remarkable decrease in miR-27 and miR-143 expressions. Studies have found that the regulatory effects of miRNAs on a target gene are generally weak (± 40% amplitude of accommodation) (Ambros, 2004). Moreover, a single miRNA can simultaneously target multiple genes, whereas one gene can be regulated by various miRNAs (Hulsmans et al., 2011). Therefore, while dietary CLA can significantly reduce miR-27 and miR-143 expressions in the adipose tissues of pigs, the effect of altered miRNA expression on the target genes PPARγ and C/EBPα may be attenuated by other miRNAs. Thus, there is a slight change in the PPARγ and C/EBPα expressions. FABP4 primarily transports intracellular free fatty acids to organelles and most likely binds to long-chain polyunsaturated fatty acids. Thus, high FABP4 expression was most likely caused by the intake of dietary CLA, which is a long-chain polyunsaturated fatty acid. Furthermore, our data analysis results demonstrated that miR-27 expression in the back and abdominal fat tissues exhibited a significant negative correlation with FABP4 expression. These results are consistent with previous reports, which showed that FABP4 is a target gene of miR-27.

In summary, CLA supplementation decreased body fat deposition in pigs and changed the expression levels of adipose tissue-specific miRNAs. These results suggested that miRNA expression in animals could be regulated by nutrients and/or nutrition level. Further, detailed studies should be conducted to determine the regulatory mechanisms involved in this process.

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