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β-carotene as a dietary factor affecting expression of genes connected with carotenoid, vitamin A and lipid metabolism in the subcutaneous and omental adipose tissue of beef cattle

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ABSTRACT. Adipose tissue in meat, especially subcutaneous fat, is not appreciated by consumers as it is considered unhealthy. The effects of β-carotene (βC) on the expression of ten genes related to carotenoid, vitamin A (VA) or lipid metabolism were evaluated in subcutaneous and omental (visceral) adipose tissue of Simmental crossbred steers receiving various βC treatments (0, 600, 1200 and 1800 mg/d). Two carotenoid oxidative cleavage genes (β-carotene-15,15'-monooxygenase (*BCMO1*) and β-carotene-9',10'-dioxygenase (*BCO2*)) were up-regulated and three VA metabolic genes (retinoid X receptor α (*RXRA*), retinal reductase (*RALDH*) and lecithin-retinol acyltransferase (*LRAT*)) were down-regulated in subcutaneous and omental adipose tissues. Gene encoding peroxisome proliferator-activated receptor γ (*PPARG*) involved in adipocyte differentiation and lipogenesis was down-regulated in both examined fat tissues. For the omental adipose tissue, the lipogenesis gene (fatty acid synthase (*FAS*)) and the two lipolysis genes (hormone-sensitive lipase (*HSL*) and adipose triglyceride lipase (*ATGL*)) were down-regulated, but another lipogenesis gene (acetyl-CoA carboxylase (*ACC*)) was up-regulated. First of all, the addition of βC in the diet may inhibit the expression of the major adipogenesis gene – *PPARG*, and increase the expression of genes involved in βC catabolism in adipose tissue of beef cattle. An effective βC dose to regulate the expression of genes connected with carotenoid, VA and lipid metabolism would be at least 600 mg/d.

Introduction

Adipose tissue is essential for whole-body energy homeostasis and can be affected by dietary nutrient levels and many organic compounds such as vitamins or amino acids. The fat of animal origin is not appreciated by consumers because it is considered to be unhealthy (Liu et al., 2014). However, the quantity and distribution of fat in the body, as well as fatty acid composition in the adipose tissue, are important contributors to various aspects of meat quality (Siebert et al., 2006). Because of the consumer's increasing awareness of nutrition for healthcare on one hand and the need of reaching a certain level of fat in the meat to guarantee its organoleptic quality on the other, a better knowledge of adipogenesis in meat of farm animals would be desirable. Therefore, understanding adipose tissue physiology and regulating its development safely and effectively have become the important aspects of meat production.

Carotenoids, that play an important role as provitamin A and antioxidants, are C40 lipophilic

pigments produced by photosynthetic organisms (Tourniaire et al., 2009). Among the hundreds of types of carotenoids, bovines mainly absorb β-carotene (βC) (Yang et al., 1992), which is also the main provitamin A carotenoid. Many but not all studies confirmed that the levels of βC or vitamin A (VA) in the diet of beef cattle can influence fat deposition and meat quality (Yang et al., 1992; Siebert et al., 2006; Gorocica-Buenfil et al., 2007; Condron et al., 2014). Previous studies on βC or VA effects in cattle were limited to their impacts on growth performance, carcass characteristics or other growth traits but rarely investigated the underlying mechanisms.

The β-carotene-15,15′-monooxygenase (BCMO1) is currently considered to be the key enzyme for the conversion of carotenoids (mainly βC) to retinoid. The product of BCMO1, retinaldehyde (RAL), could be reduced to retinol (ROH) by retinal dehydrogenases (RALDH) and then be esterified to form retinyl ester by lecithin-retinol acyltransferase (LRAT) (Shmarakov et al., 2013; Mezaki et al., 2016). Both RALDH and LRAT are important factors to predict the downstream molecules of BCMO1. Besides, the *BCMO1* promoter contains a peroxisome proliferator response element (PPRE), and when the peroxisome proliferator-activated receptor γ (PPARG) dimerizes with the retinoid X receptor α (RXRA), it binds to this site. The natural ligands for mentioned above receptors, i.e., free fatty acids (FFA) for PPARs and retinoids for RXRs, can activate *BCMO1* expression (Boulanger et al., 2003; Gong et al., 2006).

A second carotenoid metabolizing enzyme, β-carotene-9′,10’-dioxygenase (BCO2), catalyzes eccentric oxidative cleavage of carotenoids, such as βC and lycopene, at the C9′,C10′ double bonds (Gong et al., 2006). There are several other enzymes involved in lipid or carotenoids metabolism, among which acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) participate in fatty acid biosynthesis, and hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) catabolize stored triglycerides in the adipose tissue (Zimmermann et al., 2004; Lafontan and Langin, 2009).

To investigate the underlying mechanisms of βC on adipose tissue development in different anatomical locations, we analysed the expression of *BCMO1*, *PPARG*, *RXRA*, *BCO2*, *RALDH* and *LRAT* in subcutaneous and omental (visceral) adipose tissues, and the expression of *FAS*, *ACC*, *HSL* and *ATGL* in the omental adipose tissue. It will improve our understanding of how βC affects the meat quality and fat deposition processes in beef cattle.

Material and methods

The whole procedure with experimental animals was performed in strict accordance with guidelines (IACC20060101, 1 Jan, 2006) of the Institutional Animal Care and Use Committee of Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences (China).

Animals and management

To investigate the effects of supplementing βC to the diet on fat deposition in the practical production of beef cattle, 120 continental crossbred (Simmental × local yellow cattle) steers (mean live weight: 381 ± 26.01 kg) were selected from feedlots and assigned randomly to four groups supplemented with different concentrations of βC per individual (i.e., 0, 600, 1200 or 1800 mg/d) in standard basic daily ration. The composition and nutrient contents of concentrates (expressed as dry matter) are shown in Table 1. After the 15-day adaptation period, animals were restrained and fed individually during the whole 90-day experimental period. The concentrate feeding amount was controlled to 1% body weight of the steers to meet energy and major nutrients provision of the diet at about 1.3 times of maintenance requirements of beef cattle (Feng, 2000), and was adjusted once a month according to the steer's weight. Cattle were fed twice daily (06:00 and 16:00) and each dose of βC was weighed every day for each animal, mixed with concentrate and split between two feeds. For more details about animals and management see Jin et al. (2015; 2016).

Table 1. Composition and nutrient contents of concentrates (expressed as dry matter)

Ingredients	Content, %	Nutritional ingredient	Nutrient level, %
Maize	37.4	Dry matter (DM)	80.63
Wheat bran	11.7	Crude protein (CP)	15.80
Maize skin	12.0	Net energy (NE_{mf})[2], MJ/kg DM	11.78
Soybean meal	9.9	Calcium (Ca)	1.44
Palm meal	20.6	Phosphorus (P)	0.84
$CaHPO_4$	1.7		
Limestone	1.7		
Sodium bicarbonate	2.2		
NaCl	1.7		
Premix[1]	1.1		

[1] The premix contained the following components per kg of concentrate: IU: vitamin A 1,250, vitamin D_3 270, vitamin E 5; mg: manganese 3,060, zinc 14,280, iron 3,170, copper 3,040, selenium 100, iodine 180, cobalt 40; [2] NE_{mf} was a calculated value, while other values were measured

Adipose tissue sampling

Ten randomly selected individuals from each group were slaughtered in a commercial slaughterhouse at the end of the 90-day experimental period. Fat samples from omental (visceral) and subcutaneous adipose tissue taken between the 12th and 13th ribs were collected into sterile tubes and stored in liquid nitrogen for further analysis of gene expression. All ten subcutaneous and omental fat samples from each group were used for mRNA level analysis, and three randomly selected from each group for protein determination.

Western blot analysis

The protein expression levels of BCMO1, PPARγ and RXRα were analyzed by Western blot. Briefly, adipose tissue samples were frozen in liquid nitrogen and homogenized using radio immunoprecipitation assay (RIPA) buffer. For 50–100 mg of tissue, 0.5–1.0 ml of RIPA buffer was added. The homogenate was transferred to a 1.5-ml centrifuge tube and disrupted by ultrasonication on ice four times, each for 30 s at 100–200 W. The mixture was centrifuged at 12 000 *g* at 4 °C for 10 min and the total protein in the supernatant was collected. The details about the Western blot analysis have been described previously by Liu et al. (2018). Signals were detected with chemiluminesence method using the eECL Western Blot Kit (CoWin Biosciences, Inc., Beijing, China), and the images were acquired by GE ImageQuant LAS 500 imaging system (GE Healthcare Life Sciences, Uppsala, Sweden). Relative quantities of proteins were determined using Image-Pro Plus software (Media Cybernetics, Inc., Sliver Spring, MD, USA) by analyzing the sum density of each protein band image. The quantity of β-actin was used as an internal control. The density value of each sample was normalized to its β-actin density value to obtain its relative quantity value. The relative quantities of target proteins in the control group were set as one, and the values in βC treatment groups were expressed relative to this quantity.

The primary antibodies were chosen as follows: anti-BCMO1 polyclonal antibodies (1:200, sc-163736; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-PPARγ polyclonal antibodies (1:400, NB120-19481; Novus Biologicals, Littleton, CO, USA), anti-RXRα monoclonal antibodies (1:1000, LS-C80051; LifeSpan BioSciences, Inc., Seattle, WA, USA), and anti-β-actin monoclonal antibodies (1:1000, 4970S; Cell Signaling Technology, Inc., Beverly, MA, USA).

RNA extraction and qPCR

Total RNA was isolated from fat tissues samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). For each sample, 1 µg of total RNA was used for first-strand cDNA synthesis as previously described (Liu et al., 2009). Real-Time PCR (qPCR) primers were designed and synthesized (Biosune, Shanghai, China) to assay differentially expressed genes (Table 2). Because of its stability among different adipose tissues (Schoof et al., 2004), the gene encoding β-actin (*ACTB*) was used as the housekeeping gene, and its Ct values in different tissues were expressed stably at 17–18 in the present study.

Table 2. Primer sequences of genes selected for qPCR analysis

Gene	Primer (5'→3')	Accession number	Product length, bp
BCMO1	Forward: GGCTTACATTCGGGGTGTGA Reverse: CGTCCTTCGGTCGATGATGT	NM_001024559.1	88
PPARG	Forward: CACCACCGTTGACTTCTCCAG Reverse: ATACAGGCTCCACTTTGATTGC	NM_181024.2	140
RXRA	Forward: CTCAATGGTGTCCTCAAAGTG Reverse: TCAGGCAGTCCTTGTTGTCC	NM_001304343.1	199
BCO2	Forward: GAACGGAGCAACTGCACATC Reverse: TGTCTCCCCAGGGTCAGATT	NM_001101987.2	133
RALDH	Forward: TACATCGCTGCACCCCAAAT Reverse: TCCCCATTTTGTACGTCCCG	XM_005212080.4	185
LRAT	Forward: CCTGACGCACTATGGCATCT Reverse: TGAGACGCTTGTTGGAGACC	NM_177503.2	122
FAS	Forward: GTGGGCTTGGTGAACTGTCT Reverse: AGGACTTCGGGTCTGTCTCA	NM_001012669.1	112
ACC	Forward: AACGCAGGCATCAGAAGATT Reverse: CGCACTCACATAACCAACCA	NM_174224.2	119
HSL	Forward: TGGGTTTCCAGTTCACACCT Reverse: GATGCCAGTCTCGTTTCGTT	NM_001080220.1	101
ATGL	Forward: TGTTCCCCAAGGAGACGAC Reverse: GCCACGCCGATATGGTAGAC	FJ798978.1	76
ACTB	Forward: CAGCAAGCAGGAGTACGATG Reverse: AGCCATGCCAATCTCATCTC	NM_173979.3	137

qPCR was performed using a real-time thermocycler (Light Cycler 480; Roche, Mannheim, Germany) with a final reaction volume of 20 µl containing SYBR Green I (Roche, Mannheim, Germany). The cycling conditions were as follows: 95 °C for 8 min; 40 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 30 s; 95 °C for 5 s and 65 °C for 1 min. The Ct values were obtained and the gene expression levels relative to that of the *ACTB* were determined using the $2^{-\Delta\Delta(Ct)}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All data were analysed by one-way analysis of variance (ANOVA) using SPSS ver. 21.0 software (IBM, Inc., Armonk, NY, USA). Expression of all examined genes (*BCMO1*, *RXRA*, *PPARG*, *BCO2*, *RALDH*, *LRAT*, *FAS*, *ACC*, *HSL* and *ATGL*) and proteins (BCMO1, RXRα and PPARγ) in the subcutaneous and omental fat samples were analysed to assess the effects of the supplemental βC treatments. If ANOVA results were significant, Duncan's multiple-range test was performed for multiple comparisons, with $P < 0.05$ accepted as statistically significant.

Results

Gene and protein expression of BCMO1, PPARγ and RXRα in two different adipose tissues

Compared to the group without βC addition, higher mRNA levels of *BCMO1* in both subcutaneous and omental adipose tissue were observed in all groups with βC treatment ($P < 0.05$; Figures 1A and 1D). The expression of *PPARG* in both examined adipose tissues was significantly decreased in the groups that received βC supplementation ($P < 0.05$; Figures 1B and 1E). The expression of *RXRA* was decreased in subcutaneous and omental fat samples collected from beef cattle with additional βC supplementation ($P < 0.05$; Figures 1C and 1F).

The protein expressions of BCMO1, PPARγ and RXRα in both subcutaneous and omental adipose tissue were consistent with the expression pattern of the corresponding gene (Figures 2 and 3 for subcutaneous and omental adipose tissue, respectively).

Expression of genes involved in carotenoid metabolism in two different adipose tissues

BCO2 expression levels were higher in βC supplemented groups than that in the control one for both subcutaneous and omental adipose tissues ($P < 0.05$; Figures 4A and 4D).

The *RALDH* expression in subcutaneous fat was significantly lower in the group treated with 1200 mg/d βC than in the control and other supplemented groups ($P < 0.05$; Figure 4B). In omental adipose tissue, βC supplementation decreased the *RALDH* expression regardless used βC dose ($P < 0.05$; Figure 4E).

The *LRAT* expression in subcutaneous adipose tissue was significantly decreased in all groups supplemented with βC in comparison with the control group ($P < 0.05$; Figure 4C). In omental adipose

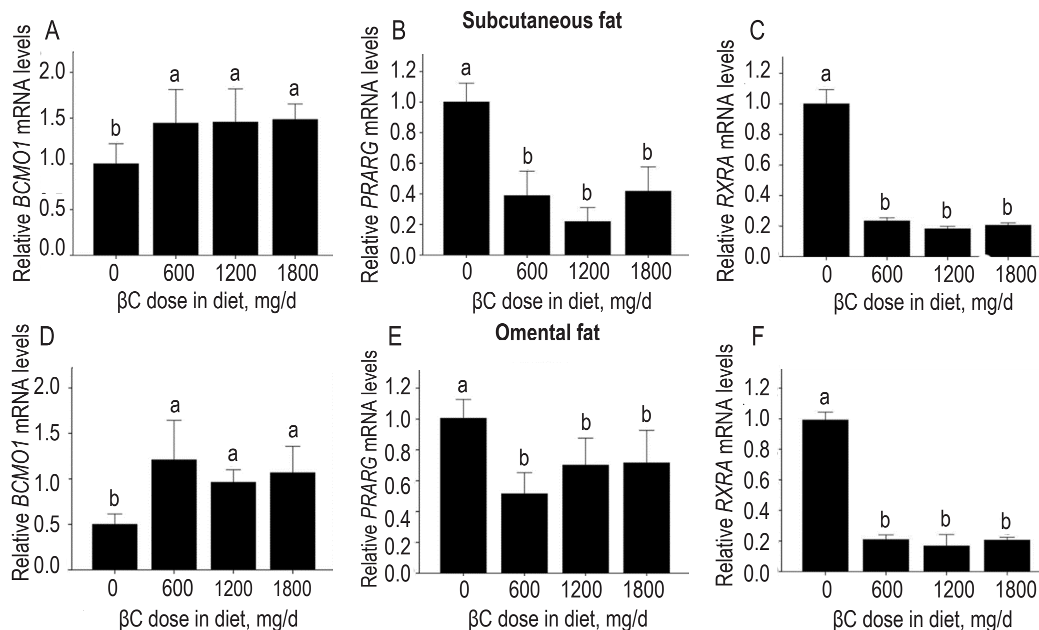

Figure 1. Expression of *BCMO1* (A and D), *RXRA* (D and E) and *PPARG* (C and F) in subcutaneous and omental fat, respectively, of beef cattle fed different doses of β-carotene (βC) (0, 600, 1200 and 1800 mg/d); bars present means ± standard error (SE), for 10 steers per group; a–b – bars with different superscripts vary significantly ($P < 0.05$)

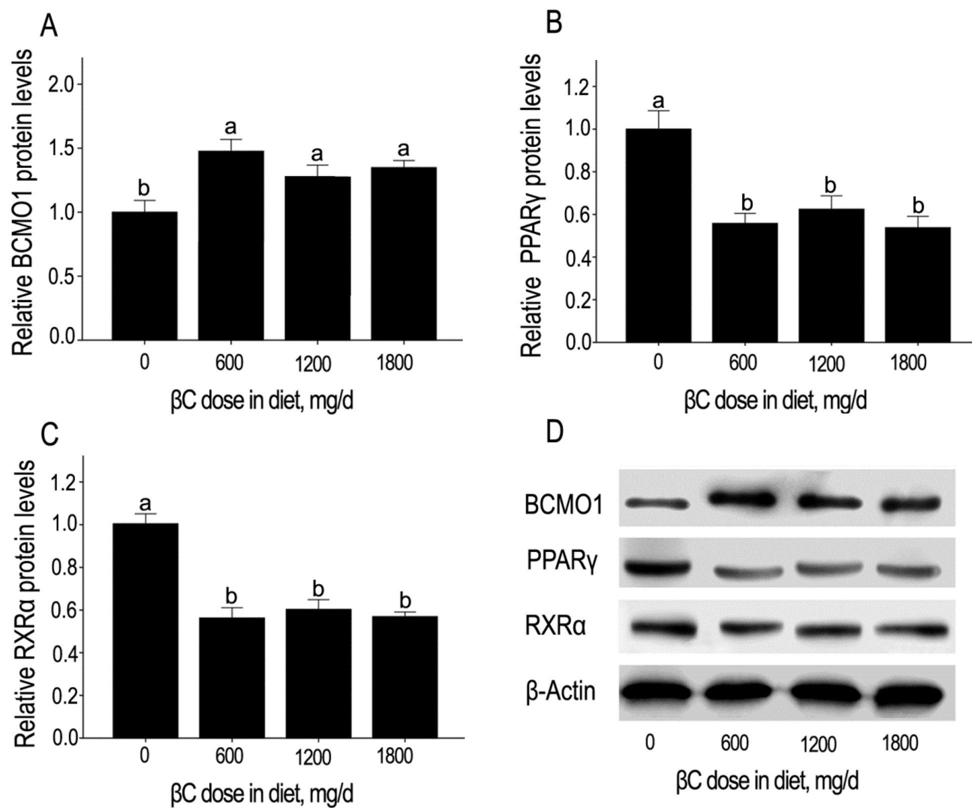

Figure 2. Expression of BCMO1 (A), PPARγ (B) and RXRα (C) protein in subcutaneous fat of beef cattle fed different doses of β-carotene (βC) (0, 600, 1200 and 1800 mg/d); Figure A, B and C: bars present means ± standard error (SE), for 3 steers per group; a–b – bars with different superscripts vary significantly ($P < 0.05$); Figure D: bands present one representative sample from each group

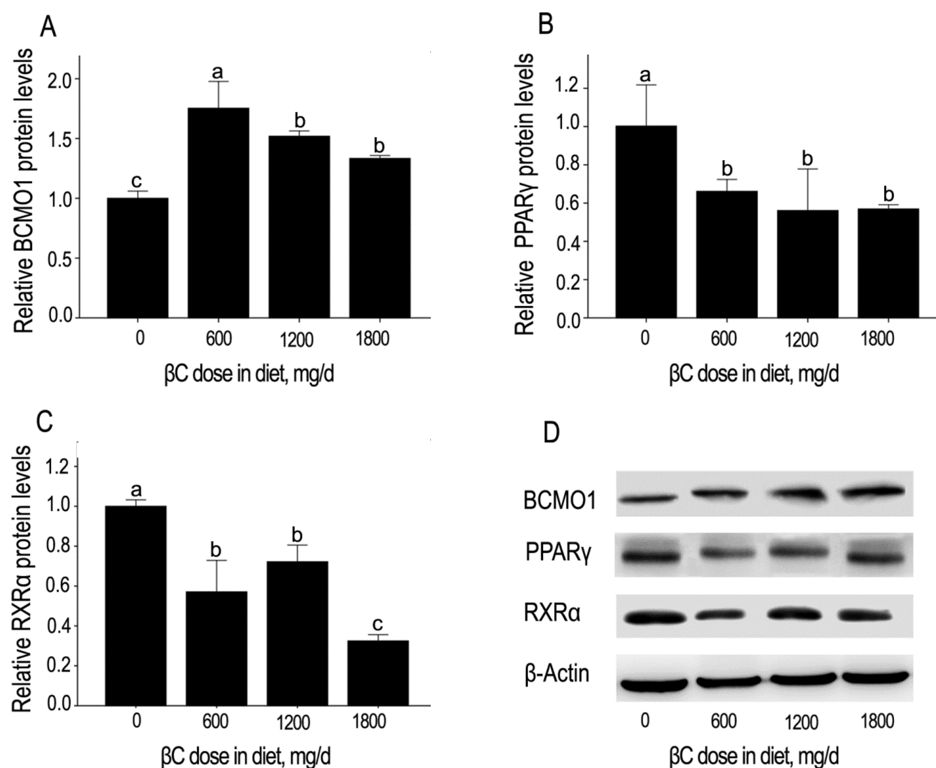

Figure 3. Expression of BCMO1 (A), PPARγ (B) and RXRα (C) protein in omental fat of beef cattle fed different doses of β-carotene (βC) (0, 600, 1200 and 1800 mg/d); Figure A, B and C: bars present means ± standard error (SE), for 3 steers per group; a–c – bars with different superscripts vary significantly ($P < 0.05$); Figure D: bands present one representative sample from each group

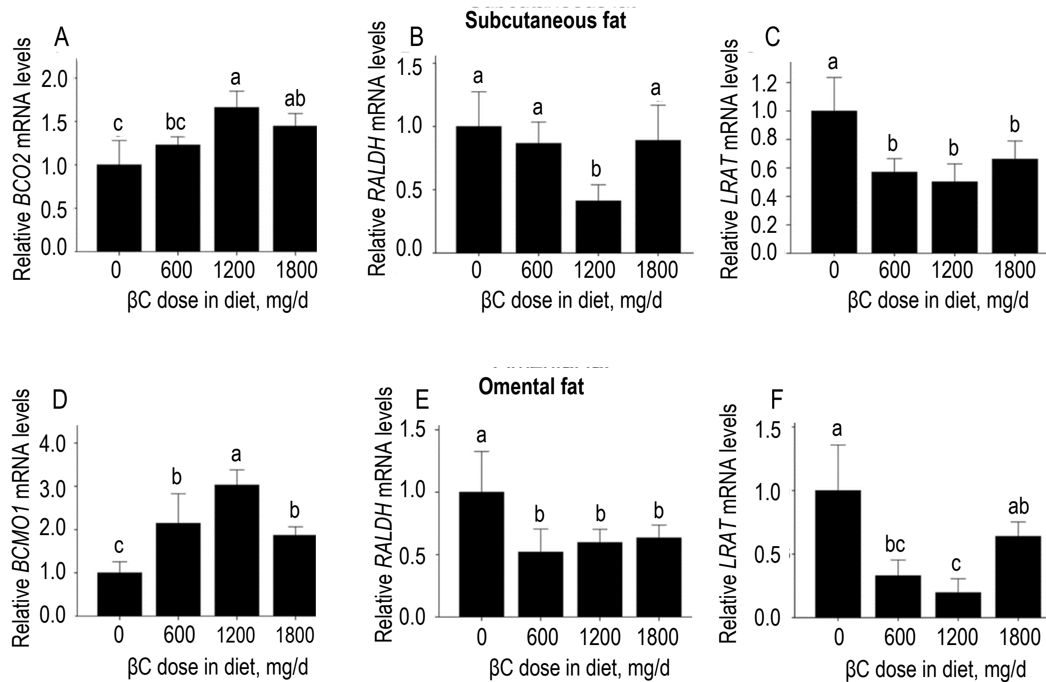

Figure 4. Expression of *BCO2* (A and D), *RALDH* (D and E) and *LRAT* (C and F) in subcutaneous and omental fat, respectively, of beef cattle fed different amounts of β-carotene (βC) (0, 600, 1200 and 1800 mg/d); bars present means ± standard error (SE), for 10 steers per group; a–c – bars with different superscripts vary significantly ($P < 0.05$)

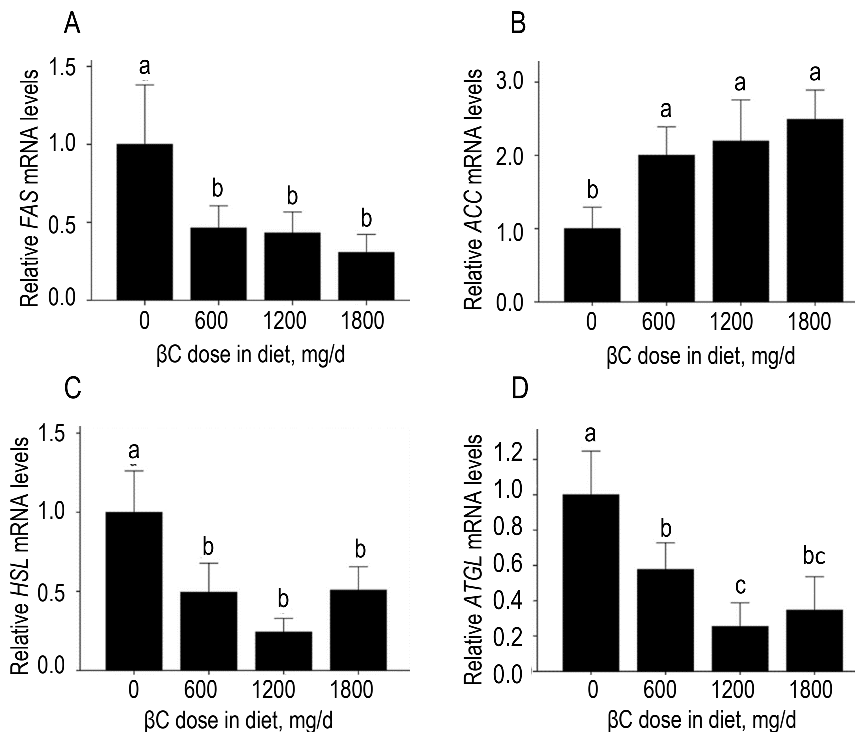

Figure 5. Expression of fat metabolism related genes (*FAS* (A), *ACC* (B), *HSL* (C) and *ATGL* (D)) in omental fat of beef cattle fed different amounts of β-carotene (βC) (0, 600, 1200 and 1800 mg/d); bars present means ± standard error (SE), for 10 steers per group;. a–c – bars with different superscripts vary significantly ($P < 0.05$)

tissue the *LRAT* expression was reduced in the βC supplemented groups at a dose of 600 and 1200 mg/d compared with the control group ($P < 0.05$; Figure 4F).

Expression of genes involved in lipid metabolism in omental adipose tissue

In omental adipose tissue, the *FAS* and *HSL* expression levels were significantly decreased in

groups supplemented with βC regardless its dose ($P < 0.05$; Figure 5A and 5C). On the other hand, the *ACC* expression was increased in all supplemented groups ($P < 0.05$; Figure 5B). The expression of *ATGL* was also down-regulated in the βC-treated groups; however the *ATGL* expression in the group with supplementation at a dose of 1200 mg/d was significantly lower from the group with supplementation at a dose of 600 mg/d ($P < 0.05$; Figure 5D).

Discussion

There is growing evidence that acting as signalling molecules βC, VA and their derivatives can exert influence on adipocyte physiology by acting on parameters related to adiposity (García et al., 2009). Similarly, the effects of βC supplementation on BCMO1, PPARγ and RXRα gene and protein expressions stated in the present study showed that there is a close relationship between carotenoids and fat metabolism in beef cattle, which was also found in our previous study (Jin et al., 2016). Adipose tissue was reported to be an important place for βC storage (Yang et al., 1992; Reynoso et al., 2004), which was also confirmed by our previous research (Jin et al., 2015). In the present study, the higher expression of *BCMO1* and *BCO2* in βC supplemented groups in subcutaneous and omental adipose tissue may be caused by higher content of βC reported by Jin et al. (2015), however the lack of differences among the supplemented groups may indicate a limited βC metabolism capacity of adipose tissue.

Some papers demonstrated that dietary βC has a repression effect on adipose tissue *via* BCMO1 (Amengual et al., 2011; Lobo et al., 2012), which was proposed to influence adipocyte physiology by contributing directly to VA production (Tourniaire et al., 2009). However, the results of the present study illustrated that supplementing βC down-regulated the VA anabolism related genes such as *RXRA*, *RALDH* and *LRAT*. It can be inferred that no inevitable connection exists between fat metabolism and VA generation. Furthermore, Ziouzenkova et al. (2007) reported that physiological concentration of retinal (one of the βC conversion products) repressed lipogenesis *in vitro* and *in vivo* through inhibition of *RXRA* and *PPARG* activation *via* their respective ligands. In the present study, caused by βC addition down-regulation of *RXRA* and *PPARG* expression was accompanied by the down-regulated expressions of *RALDH* and *LRAT*, which was consistent with the lack of differences in VA content between βC supplemented and control groups observed by Jin et al. (2015). Therefore, the repression effect of βC on lipogenesis may be more related with βC catabolism rather than VA anabolism.

Interestingly, our previous study showed that supplementing βC up to 600 mg/d could increase concentrations of βC in subcutaneous and omental fat (Jin et al., 2015). Moreover in the present study the βC supplementation up-regulated the two βC catabolism related genes *BCMO1* and *BCO2*, which illustrates the capability of adipose tissues in βC storage and catabolism.

For the lipid metabolism related genes, dietary βC exerted reverse regulation effects on fat anabolism genes *FAS* and *ACC* in the omental fat. However, our previous results showed that in subcutaneous fat expression of both these genes was down-regulated in all group with βC supplementation (Jin et al., 2016). So, it could be suggested that tissue-specific effects of βC toward lipid metabolism related genes exist in beef cattle. The reverse effects of βC on *FAS* and *ACC* expressions in the omental adipose tissue may shed new light on the result of Condron et al. (2014) who reported that omental adipose tissue (kidney, pelvic and heart fat) was not affected by dietary βC addition up to 22 000 IU/kg.

Conclusions

Supplementation of β-carotene (βC) in the diet may inhibit the expression of the major adipogenesis gene – *PPARG* and enhance the expression of βC catabolism involved genes in adipose tissue of beef cattle, which reflects a close connection between βC and lipid metabolism. The effective dose of βC to observed changes in βC, vitamin A and lipid metabolism related genes expression would be at least 600 mg/d.

Acknowledgments

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