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Betaine suppresses carnitine palmitoyltransferase I in skeletal muscle but not in liver of finishing pigs

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ABSTRACT

An experiment was conducted to investigate the effect of dietary betaine supplementation on the enzyme activity and mRNA abundance for carnitine palmitoyltransferase I (CPT I) in liver and skeletal muscle of finishing pigs. Forty-eight crossbred barrows and gilts [Duroc × (Seghers × Seghers)] weighing about 55 kg were divided into two dietary treatments, each with three replicates of eight pigs (four barrows and four gilts) per replicate. Pigs were fed a corn–soybean meal basal diet supplemented with betaine at 0 or 1250 mg/kg feed for 42 days. At trial termination, two pigs (one barrow and one gilt) weighing about 90 kg were selected from each replicate (six pigs per dietary treatment) and slaughtered for analyses. The results showed that intramuscular fat content in the *longissimus* muscle of pigs fed betaine was 23.6% higher than that of controls ($P<0.05$), whereas hepatic fat content was not affected with dietary betaine treatment. Muscle-type CPT I (M-CPT I) activity, but not liver-type CPT I (L-CPT I) activity was decreased by betaine supplementation. Furthermore, betaine supplementation reduced M-CPT I mRNA abundance by 14.6% ($P<0.05$) but did not affect L-CPT I mRNA abundance. There was a positive correlation between enzyme activity and mRNA abundance for both L-CPT I and M-CPT I ($r = 0.67$ and $r = 0.72$ for L-CPT I and M-CPT I, respectively; $P<0.05$). The study suggests that betaine may be involved in fat partitioning in pigs by reducing the activity and mRNA abundance of M-CPT I, with a resultant increase in intramuscular fat content.

1. Introduction

Betaine is a naturally occurring compound present in relatively large quantities in sugar beet and aquatic invertebrates but not present in significant quantities in most animal feedstuffs. Chemically, betaine is trimethylglycine, and the physiological function of betaine is either as an organic osmoprotectant or as a methyl donor which may partially reduce the requirements for other methyl donors (e.g., methionine, choline) and participate in lipid metabolism (Saunderson and Mackinlay, 1990; Kidd et al., 1997; Simon, 1999; Eklund et al., 2005).

The use of betaine in commercial swine diets has increased since Cadogan et al. (1993) reported a 14.8% decrease in backfat thickness of pigs fed betaine-supplemented diets. Up to now, most of the studies with betaine in pigs suggests that betaine supplementation may depress overall fat deposition, and therefore this study is focused on the potential stimulatory effects of betaine on lipid oxidation as a possible mechanism to explain fat reduction in finishing pigs. Fat deposition in adipose tissue represents a balance between fat synthesis and fat degradation (Chilliard, 1993). Feng (1996) and Huang et al. (2006, 2008) indicated that addition of betaine to the diet of finishing pigs resulted in decreased carcass fat deposition by either increasing the rate of lipolysis and (or) decreasing the rate of lipogenesis.

Carnitine palmitoyltransferase I (CPT I, *EC 2.3.1.21*), an integral outer mitochondrial membrane enzyme, catalyzes the

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initial and regulatory step in the β-oxidation of fatty acids (McGarry and Brown, 1997). However, the effect of betaine supplementation on CPT I activity has not been investigated. Therefore, the objective of the present study was to evaluate the influence of betaine supplementation on the enzyme activity and mRNA abundance for CPT I in liver (L-CPT I) and skeletal muscle (M-CPT I) of finishing pigs.

2. Materials and methods

2.1. Animals and treatments

This study was approved by the Zhejiang University Institutional Animal Care and Use Committee. Forty-eight crossbred barrows and gilts [Duroc × (Seghers × Seghers)] weighing 55.7 ± 0.48 kg were randomly allotted to two dietary treatments on the basis of body weight and ancestry, and gender was equalized across treatments. Three replications (i.e. pen) of eight pigs (four barrows and four gilts) per replicate were used for each treatment. Pigs were fed a corn–soybean meal basal diet (Table 1) with or without betaine supplementation. Supplementation was 1250 mg betaine/kg feed. The basal diets were formulated to meet or exceed the nutrient requirements of finishing pigs (NRC, 1998).

The feeding experiment lasted 42 days after a 7-day adaptation period. All pigs were housed in a curtain-sided pig barn with 3.0 × 4.5-m pens and concrete floor. Each pen was equipped with a dry/wet feeder. Feed (in meal form) and water were provided for *ad libitum* consumption throughout the experiment.

2.2. Sample collection

The diet was sampled for chemical analyses. At the end of the feeding trial, 12 pigs (six pigs from each dietary treatment with one barrow and one gilt per replicate) weighing about 90 kg were selected to collect tissue samples. Pigs were stunned by electrical shock and exsanguinated. At slaughter, liver and skeletal muscle samples were rapidly removed, snap frozen in liquid nitrogen and stored at −70 °C until subsequent analyses. Liver samples were taken from the right lobe of the liver, and skeletal muscle samples were taken from the *longissimus* muscle at the 10th-rib of the carcass.

Table 1
Composition of the basal diet, as-fed basis (g/kg).

Ingredient		Nutrient [a]	
Corn	625.0	Digestible energy (MJ/kg)	12.97
Soybean meal	190.0	Crude protein	149.1
Wheat bran	148.0	Ether extract	38.1
$CaHPO_4$	12.0	Crude fibre	29.2
Limestone	10.0	Ash	53.3
Salt	4.0	Calcium	7.6
Lysine	1.0	Phosphorus	6.1
Pre-mix [b]	10.0	Lysine	7.4
		Methionine	2.1
		Cystine	2.5
		Threonine	5.2
		Tryptophan	1.9

[a] All of the data were analysed value except for digestible energy and tryptophan that were calculated using swine NRC (1998) value.

[b] Pre-mix provided the following in mg/kg diet: Cu 25, Fe 150, Mn 50, Zn 118, I 0.8, Se 0.3, Co 1, retinol 1.95, cholecalciferol 0.045, alpha-tocopherol 30, phytylmenaquinone 1.4, thiamine 1.2, riboflavin 3.6, pyridoxine 1.4, vitamin B_{12} 0.01, biotin 0.05, D-pantothenic acid 7, folic acid 0.72, niacin 16, choline 500.

2.3. Chemical analyses

Chemical analyses of the basal diets were carried out according to Association of Official Analytical Chemists (AOAC, 1995): method no. 954.01 for crude protein, no. 920.39 for crude fat, no. 978.10 for crude fibre, no. 927.02 for calcium, no. 965.17 for phosphorus, no. 982.30 for amino acids.

All liver and muscle samples were freeze-dried and then ground through a sieve (mesh size 2 mm). Crude protein and fat content were determined according to the AOAC (1995) methods and reported on dry matter basis. All analyses were done in duplicate.

2.4. Measurement of CPT I activity

Liver mitochondria were isolated by differential centrifugation as described by Mersmann et al. (1972). Muscle mitochondria were prepared by the method of Watmough et al. (1988), as modified by Power and Newsholme (1997). Liver mitochondria isolated from 1 g tissue was suspended in 1 ml of isolation medium, while muscle mitochondria isolated from 3 g tissue was suspended in 1 ml of isolation medium. Carnitine palmitoyltransferase I activity was analysed using the method of Bieber et al. (1972). The assay was based on measurement of the initial CoA-SH formation by the 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) reaction from palmitoyl-CoA by mitochondria samples with L-carnitine. Briefly, 50 μl buffer solution (containing 116 mmol/L Tris, 2.5 mmol/L EDTA, 2 mmol/L DTNB, 0.2% Triton X-100, pH 8.0) and 50 μl mitochondria suspension (0.5–1 mg mitochondrial protein per 50 μl) was added to four semi-microcuvettes (Greiner, Germany). After 5-min preincubation at 30 °C, 50 μl palmitoyl-CoA (1 mmol/L dissolved in double distilled water) was added to three cuvettes. The fourth cuvette was used as a blank, adding 50 μl water instead of palmitoyl-CoA. The reaction was then started by adding 5 μl L-carnitine solution (1.2 mmol/L dissolved in 1 mol/L Tris, pH 8.0), immediately followed by photometric measurement at 412 nm at 30 °C (Ultrospec 2000, Pharmacia Biotech Ltd, Uppsala, Sweden) for 180 s (Bremer and Norum, 1967). Activity was defined as nmol CoA-SH released/(min mg mitochondrial protein). The protein content of the mitochondria suspension was determined according to the method of Bradford (1976).

2.5. Determination of CPT I mRNA abundance

Total RNA was extracted from frozen liver and muscle using Trizol reagent as described by the manufacturer (Invitrogen Life Technologies, Gaithersburg, MD, USA), respectively. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm. In addition, the integrity of RNA was confirmed by ethidium bromide staining of ribosomal RNA following gel electrophoresis.

Reverse transcription (RT) was performed in a 25 μl reaction volume containing 1 μg total RNA, 1.0 μg random hexamer (Promega, Madison, WI, USA), 5 μl of 5× reaction buffer, 2 μl of

Table 2
Specific primer sequences of CPT I and β-actin gene.

Gene [a]	GenBank accession no.	Primer sequences [b]	Product size (bp)	Annealing temperature (°C)
L-CPT I	AF288789	S:5′-TCATCGTGGTCGTGGGCGTCAT-3′ AS:5′-CACTGGGCGGAGCAGAGGGGC-3′	733	61
M-CPT I	AY181062	S:5′-CTGAGAGATATGGCCCCTACTG-3′ AS:5′-CCAGGTCCATGACGTAATAGTTG-3′	510	57
β-actin	BC067141	S:5′-CGGGACCTGACCGACTACCT-3′ AS:5′-GGCCGTGATCTCCTTCTGC-3′	411	57

[a] L-CPT I: Liver-type carnitine palmitoyltransferase I; M-CPT I: Muscle-type carnitine palmitoyltransferase I.
[b] S: sense primer; AS: antisense primer.

10 mmol/L dexoynucleotide triphosphates (dNTPs), 1 μl of 200 u/μl Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) was carried out in a 50 μl reaction volume containing 5 μl of 10× PCR buffer (Mg^{2+} free), 3 μl of 25 mmol/L $MgCl_2$, 1 μl of 10 mmol/L dNTPs, 1 μl of each 10 pmol/L sense and antisense primers (Table 2), 2 μl of cDNA product, and 0.5 μl of 5 u/μl Taq DNA polymerase (Promega, Madison, WI, USA). The conditions of PCR amplification were as follows: one cycle of 2 min at 94 °C, then 29 cycles of each 50 s at 94 °C denaturation, 50 s of annealing (L-CPT I 61 °C; M-CPT I and β-actin 57 °C) and 1 min extension at 72 °C, and a final extension cycle of 10 min at 72 °C. Prior to doing the semi-quantitative RT-PCR experiments, products of RT-PCR-amplified with the cDNA as template were cloned to pUCm-T vector (Promega, Madison, WI, USA) and sequenced to find if the products were consistent with target genes and then used for semi-quantitative RT-PCR. Furthermore, for semi-quantitative comparisons, the optimum PCR reaction cycles to give a linear range of amplification were also determined (data not shown, 29 cycles herein). In order to confirm that there was no significant contamination in the total RNA preparation, cDNA was synthesized and control reactions were performed in the absence of reverse transcriptase, with no further PCR bands detected. PCR-amplified products were run beside molecular weight markers on 1% (w/v) agarose gels stained with ethidium bromide. Gels were photographed using the electrophoresis gel imaging system (UVP, Upland, California, USA), and mRNA abundance for L-CPT I and M-CPT I was obtained by using the ratios of corresponding CPT I/β-actin absorption density of bands on a gel, respectively.

2.6. Statistical analyses

Data are presented as treatment means and pooled standard error of the mean. Analysis of variance was used for the analysis of the effect of the diet on the different variables (SAS, 1989). For all data, treatment, gender and replicate were included as effect. Effects were considered significant if $P<0.05$. A correlation analysis was used to relate enzyme activity with mRNA abundance for CPT I.

3. Results

3.1. Chemical composition of liver and skeletal muscle

Intramuscular fat (IMF) content in *longissimus* muscle of pigs receiving supplementary betaine was 23.6% higher ($P<0.05$) than that of pigs receiving no supplementation (Table 3). Hepatic fat content was not affected with dietary betaine treatment. Differences in crude protein content of liver and muscle were not detected between treatment groups. No interactions were present between betaine and gender ($P>0.05$).

3.2. CPT I activity in liver and skeletal muscle

As shown in Table 4, an effect of betaine on the activity of L-CPT I was not observed, but the activity of M-CPT I in the betaine-supplemented group was 11.1% lower ($P<0.05$) than that in the unsupplemented group. There were no interactions between betaine and gender ($P>0.05$).

Table 3
Effect of dietary betaine supplementation on growth performance, carcass characteristics, chemical composition of liver and muscle of finishing pigs.

Item	Betaine (mg/kg feed)		Gender		S.E.M	Significance [c]
	0	1250	Barrow	Gilt	$n = 6$	
Initial weight, kg	55.56	55.85	55.90	55.52	0.49	NS [d]
Final weight, kg	89.10	91.23	90.86	89.47	0.64	Betaine
Average daily gain, g	798.50	842.22	832.39	808.24	11.45	Betaine
Feed/gain	3.11	3.03			0.02	NS
Carcass lean percentage, %	59.70	62.80	60.51	61.98	0.38	Betaine
Carcass fat percentage, %	19.35	16.82	18.69	17.47	0.31	Betaine
Average backfat thickness [a], cm	2.33	2.09	2.26	2.17	0.07	Betaine
Hepatic crude protein [b]	772.6	791.2	779.6	784.2	13.08	NS [d]
Hepatic fat content [b]	70.7	60.2	67.6	63.4	4.85	NS [d]
Muscular crude protein [b]	861.7	870.9	867.6	865.0	11.95	NS [d]
Muscular fat content [b]	84.3	104.2	90.2	98.3	6.20	Betaine

[a] Average backfat thickness was determined by averaging the backfat thickness at the first rib, last rib, and last lumbar vertebrae.
[b] Units are g/kg dry matter.
[c] Significance: $P<0.05$.
[d] NS: Not significant ($P>0.05$).

Table 4
Effect of dietary betaine supplementation on enzyme activity of carnitine palmitoyltransferase I (CPT I) in liver and skeletal muscle of finishing pigs.

Item	Betaine (mg/kg feed)		Gender		S.E.M	Significance [d]
	0	1250	Barrow	Gilt	n = 6	
L-CPT I activity [a, b]	1.89	1.97	1.93	1.92	0.06	NS [e]
M-CPT I activty [b, c]	0.81	0.72	0.77	0.76	0.03	Betaine

[a] L-CPT I: live-type carnitine palmitoyltransferase I.
[b] Units are nmol CoA-SH/min·mg protein.
[c] M-CPT I: muscle-type carnitine palmitoyltransferase I.
[d] Significance: $P<0.05$.
[e] NS: Not significant ($P>0.05$).

3.3. CPT I mRNA abundance in liver and skeletal muscle

Dietary betaine did not affect L-CPT I mRNA abundance, but it decreased M-CPT I mRNA abundance by 14.6% ($P<0.05$) (Table 5). In addition, there were relatively high correlations between enzyme activity and mRNA abundance for both L-CPT I and M-CPT I (r = 0.67 and r = 0.72 for L-CPT I and M-CPT I, respectively; $P<0.05$). No interactions were observed between betaine and gender ($P>0.05$).

4. Discussion

In our previous study, daily gain was increased and carcass characteristics were improved by dietary betaine supplementation (Huang et al., 2006). Those data are consistent with the results of some studies, which indicate that betaine may decrease fat deposition and increase carcass lean in pigs (Feng, 1996; Casarin et al., 1997; Matthews et al., 2001; Yan, 2001). However, other reports show that betaine does not affect daily gain and carcass traits (Matthews et al., 1998; Øverland et al., 1999). More research is required to confirm the influence of betaine on daily gain and carcass characteristics of pigs.

In order to evaluate whether the reduction of carcass fat was only in subcutaneous tissues or it affected other parts of the carcass, hepatic fat content and IMF content in *longissimus* muscle were measured in this study. The results showed that hepatic fat content tended to decrease in betaine-fed pigs (P = 0.12), whereas IMF content in *longissimus* muscle of betaine-fed pigs was increased. This is in agreement with Feng (1996) and Ma et al. (2000), who reported that the addition of 1000, 1250, 1500, 1750 mg/kg betaine to swine diets markedly increased IMF content in *longissimus* muscle. The addition of betaine also reduces carcass fat content and affects the distribution of carcass fat in other animal such as broilers (Saunderson and Mackinlay, 1990) and fish (Virtanen et al., 1989). However, adipose tissue is considered to be the primary site of *de novo* fatty acid synthesis in pigs (O'Hea and Leveille, 1969). IMF deposits relatively later than subcutaneous fat (Lee et al., 1973; Lee and Kauffman, 1974). So, while reduction of subcutaneous fat may be considered as a positive effect of betaine, the increase of IMF in pigs fed betaine needs further investigation.

Acting as a methyl donor, betaine may be integrally involved in lipid metabolism via its role on phosphatidylcholine synthesis and in fatty acid oxidation via its role on carnitine synthesis. Dietary carnitine supplementation has been shown to decrease carcass fat content in pigs (Owen et al., 1996). Betaine has been reported to increase carnitine concentrations in liver and muscle of pigs (Feng, 1996), implicating an effect of betaine on the reduction of carcass fat content in pigs. Carnitine palmitoyltransferase I is a major regulatory enzyme of lipid metabolism. Enzyme analysis showed that dietary betaine supplementation caused a decrease in the activity of M-CPT I, but it had no apparent effect on the activity of L-CPT I, suggesting that the decreased fatty acid oxidation in skeletal muscle can be one of the factors contributing to the increased IMF content in betaine-fed pigs. It is well established that long-term changes in CPT I activity are based on changes at the transcriptional level (McGarry and Brown, 1997). This study is the first to examine the effect of betaine on CPT I mRNA abundance in pigs. The result indicated that dietary betaine indeed decreased M-CPT I mRNA abundance but did not affect L-CPT I mRNA abundance. Furthermore, there was a significant correlation between enzyme activity and mRNA abundance for both L-CPT I and M-CPT I, implying that CPT I may be regulated by betaine at a pre-translational step.

Carnitine palmitoyltransferase I catalyzes the conversion of long-chain acyl-CoAs to acylcarnitines in the presence of L-carnitine. The reaction catalyzed by CPT I is the first rate-limiting step in fatty acid oxidation in all tissues and is highly regulated by its physiological inhibitor, malonyl-CoA (McGarry and Brown, 1997). However, there are two isoforms of CPT I referred to as L-CPT I and M-CPT I based on the tissues of origin, liver (L) and muscle (M), in which the expression of CPT I genes was first studied. Furthermore, the two isoforms of CPT I differ markedly in their kinetic characteristics, namely the *K*m for

Table 5
Effect of dietary betaine supplementation on mRNA abundance of carnitine palmitoyltransferase I (CPT I) in liver and skeletal muscle of finishing pigs.

Item	Betaine (mg/kg feed)		Gender		S.E.M	Significance [c]
	0	1250	Barrow	Gilt	n = 6	
L-CPT I mRNA abundance [a]	0.71	0.85	0.75	0.81	0.07	NS [d]
M-CPT I mRNA abundance [b]	0.41	0.35	0.39	0.37	0.02	Betaine

[a] L-CPT I: live-type carnitine palmitoyltransferase I.
[b] M-CPT I: muscle-type carnitine palmitoyltransferase I.
[c] Significance: $P<0.05$.
[d] NS: Not significant ($P>0.05$).

carnitine and the sensitivity to malonyl-CoA inhibition (McGarry and Brown, 1997). Pig L-CPT I has a low Km for carnitine and possesses a higher sensitivity to malonyl-CoA inhibition, while pig M-CPT I has a high Km for carnitine and shows low sensitivity to malonyl-CoA inhibition (Nicot et al., 2001; Relat et al., 2004). In conjunction with the mitochondrial acetylcarnitine transferase and acetylcarnitine translocase system, there is a significant positive correlation between acetyl-CoA and malonyl-CoA levels (Saddik et al., 1993). Betaine has been reported to increase carnitine concentrations in liver and muscle of finishing pig (Feng, 1996), thus stimulating the mitochondrial acetylcarnitine transferase and acetylcarnitine translocase pathway by which acetyl-CoA can be transported from the mitochondria to the cytosol. As a result, increased cytosolic acetyl-CoA levels could stimulate acetyl-CoA carboxylase and malonyl-CoA production, leading to a decrease in CPT I activity (Saddik et al., 1993). The different response of L-CPT I and M-CPT I activities to dietary betaine supplementation could be attributed to the marked difference between the two CPT I isoforms in their *K*m for carnitine and the sensitivity to malonyl-CoA inhibition. In addition, betaine decreased M-CPT I mRNA abundance but not affect L-CPT I mRNA abundance, which could also contribute to be the different response of L-CPT I and M-CPT I activities to dietary betaine supplementation. As M-CPT I activity decreased, fatty acid oxidation in skeletal muscle decreased (Kudo et al., 1995), inducing an increase in incorporation of fatty acids into neutral lipids and subsequently causing an increased IMF content (Rasmussen and Wolfe, 1999). This indicates that betaine can alter lipid partitioning in finishing pigs.

Fatty acid oxidation depends to a large extent on circulating fatty acid concentration. Serum free fatty acids (FFA) are mainly generated by the hydrolysis of triacylglycerols in adipose tissue, and their concentration reflects the lipolytic activity in adipose tissue (Mersmann and MacNeil, 1985). In our previous studies, hormone-sensitive lipase activity in adipose tissue and serum FFA concentration was significantly increased in pigs fed betaine (Huang et al., 2006), implying that betaine exerted a lipolytic effect on adipose tissue. In pigs, there was no correlation between IMF content and hormone-sensitive lipase gene expression (Chen, 2003). Elevated FFA concentration could enhance uptake of FFA from the bloodstream to muscle tissue, with a resultant increase in fat deposition in muscle tissue (Rasmussen and Wolfe, 1999). This is consistent with the finding that backfat thickness decreased and IMF content increased with betaine supplementation.

In conclusion, the present study indicated that addition of 1250 mg/kg betaine to diets of finishing pigs increased IMF content, which was associated with decreased activity and suppressed mRNA abundance of M-CPT I. Betaine may therefore be involved in fat partitioning in pigs. Further studies are required to elucidate the mechanism by which betaine regulate fat deposition in pigs.

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