L-CARNITINE SUPPLEMENTATION TO GESTATING GILTS ALTERS THE IGF AXIS IN PORCINE EMBYRONIC MYOBLASTS[1]

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Summary

We determined the effects of supplemental L-carnitine on the insulin-like growth factor (IGF) system in porcine embryonic myoblasts (PEM) from gilts. Forty gilts (BW = 303.6 lb) were allotted to 1 of 4 treatments that were arranged in a 2 × 2 factorial, with main effects of L-carnitine (0 or 50 ppm) and day of gestation (55 or 70). All gilts were fed 3.86 lb/day and a top-dress containing either 0 or 50 ppm of L-carnitine, starting on the first day of breeding and continuing through the allotted gestation length. At d 55 or 70 of gestation, fetuses were removed for isolation of PEM from the hind-limb muscles. Real-time quantitative PCR was used to determine growth factor messenger RNA (mRNA) expression in cultured PEM at 72-, 96-, 120-, and 144-h after plating. Flow cytometry was used to analyze percentage of myogenic cells with a myoblast/myotube-specific monoclonal antibody 5.1H11, and for determination of cell cycle stage. There was no treatment differences (P>0.10) for the expression of IGF-I, IGF-II, or IGFBP-5 mRNA levels. But PEM isolated from fetuses collected from gilts fed diets with L-carnitine had lower (P = 0.08) IGFBP-3 mRNA levels, compared with levels in the controls. Myoblasts isolated from fetuses from gilts fed diets with added L-carnitine had greater (P = 0.09; 8.8%) 5.1H11 monoclonal antibody attachment, compared with the controls, after 72 hours in culture (91.8% vs. 87.4%). Although not significant (P = 0.31), the total number of PEM in the S phase of the cell cycle was 4.7% greater in PEM collected from fetuses obtained from gilts fed diets with L-carnitine, compared with numbers from the control-fed gilts (37.5% vs. 34.2%). These data suggest that L-carnitine influences the IGF system, stage of the cell cycle, and recognition of muscle development, resulting in enhanced proliferation and delayed differentiation of PEM.

(Key Words: Insulin-like Growth Factor, Insulin-like Growth Factor Binding Proteins, L-carnitine, Messenger RNA, Myoblasts, Pigs.)

Introduction

The addition of L-carnitine to gestation diets has positive effects on sow reproductive performance. Specifically, providing supplemental L-carnitine to gestating sows increases average pig weight and litter weight at birth. This may be due to L-carnitine's role in β-oxidation, glucose disposal, and carbohydrate metabolism. Other researchers have alluded to the effect of L-carnitine on the insulin-like growth factor system.

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[1]The authors thank Lonza, Inc., Allendale, NJ, for their financial support.
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Insulin-like growth factors (IGF) and insulin-like growth factor binding proteins (IGFBP) stimulate cellular proliferation and differentiation of myogenic cells. These components of the IGF system are involved in several aspects of fetal growth and development, and seem to be modulators of muscle development in the pig fetus.

In previous data, we reported an increase in fetal weight at d 70 of gestation in fetuses collected from gilts fed diets with supplemental L-carnitine, and we have observed an increase in IGF-I, IGFBP-3, and IGFBP-5 mRNA expression in the endometrium of gilts fed diets containing supplemental L-carnitine. In addition, we observed a decrease in hepatic IGF-I expression in fetuses collected from gilts fed diets with supplemental L-carnitine. Changes in IGF-I, IGFBP-3, and -5 in the maternal endometrium and IGF-I in fetal hepatic tissue provide support for an altered maternal-fetal exchange of IGF system components due to supplementation of L-carnitine to the gilt. This may or may not explain the increased weight of pigs born to dams fed supplemental L-carnitine.

During pregnancy, growth factors and binding proteins play a role in fetal muscle development in the pig. Although data are limited, studies have observed decreased levels of IGF-II, IGFBP-3, and myogenin in PEM collected from fetuses from sows fed supplemental L-carnitine up to d 55 of gestation. Further evidence to support enhanced proliferation of porcine myogenic cells is the increase in the cross-sectional area of the semitendinosus, increased primary muscle fiber numbers, and a lower secondary:primary fiber ratio at birth in offspring of sows fed diets containing supplemental L-carnitine. Therefore, our experiment was designed to determine the effects of providing supplemental L-carnitine to gestating gilts on fetal muscle development *in vitro* by using real-time quantitative polymerase chain reaction (PCR) techniques and flow cytometry.

Procedures

All animal procedures used in this study were reviewed and approved by the Kansas State University Animal Care and Use Committee. Forty gilts (PIC, Franklin, KY; L327 × 1050; BW = 303.6 lb) were artificially inseminated (PIC; MQ 280) 12, 24, and 36 h after the onset of the second observed estrus. Gilts were randomly allotted to 1 of 2 dietary treatments and 1 of 2 harvesting dates (d 55 or 70 of gestation), based on weight at breeding. All gilts were fed a corn-soybean meal gestation diet (Table 1) once daily (3.86 lb/day) and received a 50-g top-dress containing either 0 (control, n = 30) or 88 mg (equivalent to approximately 50 ppm on an as-fed basis) of L-carnitine (Carniking 10; 10% L-carnitine, Lonza Group, Inc., Allendale, NJ, n = 29) from d 1 to d 54 or 69 gestation.

Gilts were harvested on d 55 or 70 of gestation. Gilts were allowed *ad libitum* access to water until harvest. Gilts were harvested by electrical stunning, followed by exsanguination. A mid-lateral incision was made to gain access to the abdominal cavity. The ovarian pedicles and uterine stump, at the level of the cranial cervix, were cut for removal of the uterus. Once the uterus was removed, the number of fetuses was determined on both sides.

The hindlimb muscles from the right side of the fetus were aseptically excised, washed, minced with a scissor, and pooled for each of the gilts. All cultures were maintained at 37°C, 5% CO_2, and 95% air in a water-saturated environment. After a 24-h attachment period, the plates were rinsed three times with 2.5 mL of DMEM. At 72, 96, 120, and 144 h after plating, total RNA was isolated from the cells on the 29.26 cm^2 plates (Absolutely RNA Microprep kit, Stratagene, La Jolla, CA). The concentration of total RNA was determined at an absorbance of 260 nm. TaqMan reverse transcription reagents and MultiScribe reverse transcriptase (Applied

Biosystems, Foster City, CA) were used to produce cDNA from 1 μg of total RNA. Random hexamers were used as primers in cDNA synthesis.

Table 1. Diet Composition Fed During Gestation (As-fed Basis)[a]

Item	Amount
Ingredient, %	
Corn	81.22
Soybean meal, 46.5%	14.55
Monocalcium phosphate, 21% P	2.03
Limestone	1.05
Salt	0.50
Vitamin premix[b]	0.25
Trace mineral premix[c]	0.15
Sow add pack[d]	0.25
Total	100.00
Calculated Analysis	
Lysine, %	0.65
ME, Mcal/kg	3.27
Protein, %	13.7
Calcium, %	0.85
Phosphorus, %	0.75
Available P, %	0.48

[a]Gestation feeding levels of 1.75 kg/d, with a top-dress providing either 0 or 50 ppm added L-carnitine.

[b]Supplied per kilogram of diet: 11,025 IU of vitamin A, 1,654 IU of vitamin D_3, 55.1 mg of niacin, 44.1 IU of Vitamin E, 33.1 mg of pantothenic acid, 9.9 mg of riboflavin, 4.4 mg of vitamin K (menadione), and 0.04 mg of vitamin B_{12}.

[c]Supplied per kilogram of diet: 165 mg of Zn (oxide), 165 mg of Fe (sulfate), 39.7 mg of Mn (oxide), 16.5 mg of Cu (sulfate), 0.30 mg of I (as Ca iodate), and 0.30 mg of Se (as Na selenite).

[d]Supplied per kilogram of diet: 551.3 mg of choline, 15.2 mg pyridoxine, 1.65 mg of folic acid, and 0.22 mg of biotin.

Real-time quantitative-PCR was used to measure the quantity of mRNA for IGF-I, IGF-II, IGFBP-3, and IGFBP-5 and 18S rRNA in total RNA isolated from cell cultures. Measurement of the relative quantity of cDNA was carried out by using TaqMan Universal PCR Master Mix (Applied Biosystems). Sequences for primers and probes for IGF-I, IGF-II, IGFBP-3, and IGFBP-5 are presented in Table 2. Commercially available eukaryotic 18S rRNA primers and probes were used as an endogenous control (Applied Biosystems; Genbank Accession no. X03205).

Relative expressions of mRNA for IGF-I, IGF-II, IGFBP-3, and IGFBP-5 were normalized to the 18S rRNA endogenous control by using the Δ-CT method, and were expressed in arbitrary units.

For analysis of myogenic cells, we analyzed muscle cell preparations by using a myoblast/myotube-specific monoclonal antibody 5.1H11 obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA).

The statistical analysis for gene expression was performed as a repeated measure by using the MIXED Procedure of SAS. Fixed effects of treatment, day of gestation, and hour after plating were included in the model, with hour after plating as the repeated measure. Kenward-Roger adjustment was used for the degrees of freedom. Flow cytometric analysis was performed by using the MIXED Procedure of SAS. The model contained the effects of treatment, day of gestation, and their interaction. Kenward-Roger was used to adjust the degrees of freedom.

Results and Discussion

Primary mononucleated myoblasts or muscle cells are destined to have three specific roles. They may differentiate into primary muscle fibers, promote secondary muscle fiber development, or may be reserved as a population of satellite cells aiding in skeletal muscle hypertrophy in the post-natal animal. The IGF system is instrumental in skeletal muscle development, specifically muscle cell proliferation and differentiation.

In our study, we found IGF-I and IGFBP-3 mRNA expression increased in PEM as day of gestation increased from day 55 to 70 (65%

and 50%, respectively; Table 3). Insulin-like growth factor-I and IGFBP-3 play a role in proliferation and terminal differentiation of PEM. As day of gestation increased from day 55 to day 70 of gestation, IGF-I mRNA expression increased 70%, and IGFBP-3 mRNA expression increased 72%, in skeletal muscle tissue. In our experiment, we found IGFBP-3 mRNA expression was reduced at 96 h after plating, suggesting that the initiation of terminal differentiation of PEM began at 96 h after plating. In addition, IGF-I and IGF-II mRNA levels were found to be increased at 120 h after plating. We found that levels of IGFBP-3 mRNA were increased in the control gilts at 120 h after plating. Other researchers have found, at 120-h after plating, that IGFBP-3 mRNA expression was reduced in differentiating porcine embryonic cell cultures. The increase in IGF-I and IGF-II mRNA levels at 120 h and the reduced IGFBP-3 mRNA levels at 96 h that we observed confirm the roles of IGF-I, IGF-II, and IGFBP-3 mRNA in proliferation and terminal differentiation of PEM. Therefore, we conclude that the reduced levels of IGFBP-3 mRNA we observed in the L-carnitine cultures, compared with those of the controls, are a result of PEM increased proliferative capacity, but delayed differentiation. This suggests that more nuclei may be available for muscle fiber development.

Data are limited on the effects of L-carnitine on IGF system changes in skeletal muscle development during fetal growth. In the current study, we found that mononucleated myoblasts isolated from hindlimb muscles of fetuses had changes in IGFBP-3 gene expression when their dams were fed diets with L-carnitine. Specifically, IGFBP-3 gene expression decreased 48% when L-carnitine was added to the diets.

We used flow cytometry analysis to determine if L-carnitine affected the percentage of PEM positive for the monoclonal antibody 5.1H11 and the stage of the cell cycle in proliferating myoblasts. The PEM isolated from fetuses obtained from gilts fed diets containing supplemental L-carnitine had 4.7% greater adhesion of 5.1H11. This indicates that the PEM isolated from fetuses from gilts fed diets with L-carnitine had a more enriched population of myogenic cells. This may suggest that L-carnitine has additional roles in fetal development, although direct conclusions cannot be drawn.

Similar observations were noted for flow cytometric analysis of the percentage of cells in the S phase of the cell cycle. Porcine embryonic myoblasts collected from fetuses from gilts fed diets with supplemental L-carnitine had 8.8% more PEM in the S phase of the cell cycle. A fluorescence-activated cell sorter determined this. Mitosis, the process of nuclear division, contains four distinct cycles, of which, in the S phase, synthesis of DNA occurs. In our study, 37.5% of the population of PEM collected from the fetuses from the gilts fed supplemental L-carnitine were in the S phase, and only 34.2% of the control PEM were in the S phase.

Providing supplemental L-carnitine to gestating gilts altered the IGF axis in PEM collected from fetuses. Specifically, we observed a decrease in IGFBP-3 mRNA expression in PEM collected from fetuses from gilts fed L-carnitine. Insulin-like growth factor binding protein-3 plays a role in terminal differentiation of PEM. We believe that providing supplemental L-carnitine to the dam suppresses the expression of IGFBP-3 in PEM, therefore enhancing muscle proliferation and suppressing differentation.

Table 2. Primers and Probes Used for Real-time Quantitative PCR

Gene	Genbank Accession Number		Sequence
IGF-I	M31175	Forward	TCTTCTACTTGGCCCTGTGCTT
		Reverse	GCCCCACAGAGGGTCTCA
		Probe	6FAM-CCTTCACCAGCTCTGCCACGGC-TAMRA
IGF-II	X56094	Forward	CCGGACAACTTCCCCAGATA
		Reverse	CGTTGGGCGGACTGCTT
		Probe	6FAM-CCCGTGGGCAAGTTCTTCCGC-TAMRA
IGFBP-3	AF085482	Forward	AGCACGGACACCCAGAACTT
		Reverse	CGGCAAGGCCCGTATTC
		Probe	6FAM-TCCTCTGAGTCCAAGCGCGAGA-TAMRA
IGFBP-5	U41340	Forward	GGCAGAGGCCGTGAAGAAG
		Reverse	CAGCTCCCCCCACGAACT
		Probe	6FAM-CCGCAGAAAGAAGCTGACCCAGTCC-TAMRA

Table 3. Growth Factor Messenger RNA Levels in Porcine Embryonic Myoblasts at Day 55 of Gestation[a]

	Hour After Plating								
	72		96		120		144		
	L-carnitine, ppm								
Gene	0	50	0	50	0	50	0	50	SED
IGF-I	0.13	0.21	0.17	0.05	0.30	0.35	0.18	0.30	0.277-0.330
IGF-II	199.8	179.0	58.7	133.9	415.6	558.9	123.7	568.5	353.87-387.62
IGFBP-3	40.8	13.9	1.8	0.3	5.0	1.9	14.0	16.2	15.92-16.38
IGFBP-5	147.3	69.5	74.3	27.9	47.4	69.8	45.6	57.2	87.88-93.50

[a]Gene expression levels are expressed in arbitrary units (millions).

Table 4. Growth Factor Messenger RNA Levels in Porcine Embryonic Myoblasts at Day 70 of Gestation[a]

	Hour After Plating								
	72		96		120		144		
	L-carnitine, ppm								
Gene	0	50	0	50	0	50	0	50	SED
IGF-I	0.42	0.29	0.22	0.41	0.87	1.01	0.65	0.98	0.277-0.298
IGF-II	862	41,127	599	41,017	3,655	42,280	634	41,508	29211-30,014
IGFBP-3	43.6	5.7	6.0	3.9	32.7	18.7	41.6	35.1	15.92-16.67
IGFBP-5	146.2	62.3	52.2	82.9	376.9	121.3	53.7	55.7	87.88-101.23

[a] Gene expression levels are expressed in arbitrary units (millions).

Table 5. Statistical Analysis of Growth Factor Messenger RNA Levels in Porcine Embryonic Myoblasts at Day 55 and 70 of Gestation[a]

	Probability, P <						
	TRT	Day	Hour	TRT × Day	TRT × Hour	Day × Hour	TRT × Day × Hour
IGF-I	0.58	0.01	0.01	0.73	0.84	0.29	0.83
IGF-II	0.34	0.32	0.01	0.35	0.33	0.07	0.56
IGFBP-3	0.08	0.07	0.01	0.54	0.21	0.28	0.99
IGFBP-5	0.28	0.27	0.10	0.56	0.48	0.11	0.24

Table 6. Flow Cytometric Analysis

	Day of Gestation							
	55		70					
	L-carnitine, ppm[b]					Probability, P <		
Item	0	50	0	50	SED	Trt[c]	Day[d]	Trt × Day
5.1H11, %	84.1	89.4	90.8	94.2	2.92-3.63	0.09	0.20	0.77
Cell cycle stage, %								
G_1	60.1	55.7	56.9	55.8	3.42-3.82	0.44	0.67	0.65
S	34.0	37.7	34.4	37.4	2.84-3.60	0.31	0.99	0.91
G_2	6.01	7.29	8.72	7.38	1.74-1.95	0.99	0.45	0.48