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L-Carnitine Reduces Obesity Caused by High-Fat Diet in C57BL/6J Mice

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Abstract This study evaluated the effects of carnitine supplementation on obesity caused by a high-fat diet in C57BL/6J mice. The mice were fed a normal diet (ND), high-fat diet (HD), or carnitine-supplemented (0.5% of diet) high-fat diet (HDC) for 12 weeks. The results showed that body weight, energy intake, and feed intake were lower in the HDC group than the control groups. Acid-soluble acylcarnitine (ASAC), acid-insoluble acylcarnitine (AIAC), and total carnitine (TCNE) in the serum and liver were significantly higher in the HDC group. Hepatic carnitine palmitoyl transferase-I activity was significantly higher in the HDC group than the control groups. Acyl-coA synthetase (ACS) and carnitine palmitoyl transferase-I (CPT-I) mRNA expression in the liver was highest in the HDC group, however hepatic acetyl-coA carboxylase (ACC) mRNA expression in this group was lowest. Serum leptin levels and abdominal fat weight were lowest in the HDC group. We concluded that L-carnitine supplementation diminished the risk of obesity caused by a high-fat diet.

Keywords: L-carnitine, high-fat diet, obesity, leptin, carnitine palmitoyl transferase-I (CPT-I) activity

Introduction

Obesity is a serious, social, and public health challenge that is associated with lifestyle factors such as high-fat diets and decreased exercise, and is considered one of the most intractable health problems (1, 2). The effects of obesity are not limited to being overweight, but are also associated with increased risk for many chronic diseases, such as diabetes mellitus (3), asthma (4), hypertension, and coronary arteriosclerosis (5). Reducing the incidence of obesity is an important public health goal.

L-Carnitine (L-beta-hydroxy-gamma-N-trimethylamino-butyric acid) is required during long-chain fatty acid metabolism. It is a trimethylamine biosynthesized from lysine and methionine in the liver. L-Carnitine is essential for the transport of long-chain fatty acids from the cytosol into the mitochondrial matrix, where long-chain fatty acids are oxidized, resulting in the production of ATP, with the help of L-carnitine (6-8). Fatty acids enter mitochondria as acyl-carnitines with the help of a carnitine transporter (9). In streptozotocin-induced diabetic rats, oral administration of L-carnitine normalized serum carnitine, heart rate regulation and left ventricular size (10). Moreover, when we administered carnitine to streptozotocin-induced diabetic rats, carnitine levels, which are reduced in diabetes, were restored to normal (11).

Many studies have shown the effects of L-carnitine on exercise or specific metabolites. For example, carnitine homeostasis is perturbed during exercise, which provides the theoretical basis for carnitine supplementation to improve exercise performance in healthy humans (12). However, little is known about the effect of dietary carnitine supplementation on obesity. A previous study demonstrated that L-carnitine inhibits lipogenesis in preadipocyte 3T3-L1 cells, suggesting that L-carnitine resists weight gain (13). Five tenth % of carnitine in diet decreased serum triglyceride levels in mice fed a high fat diet (14). In C57BL/6J mice, reducing the number of kilocalories consumed from a high-fat diet attenuates, but does not prevent, the development of type-II diabetes and obesity (15). In this study, we investigated the effects of L-carnitine supplementation on obese mice with type-II diabetes. This study confirmed the relationship between dietary carnitine and obesity in C57BL/6J mice, a strain with a propensity for high-fat diet-induced obesity, as shown by carnitine status and obesity indexes, such as serum lipid profiles, leptin levels, and the fat weight of each organ.

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Materials and Methods

Animals and diets Thirty male C57BL/6J mice, aged 4 weeks, were purchased from Charles River Laboratories (Tokyo, Japan). The animals were adapted to a chow diet for 1 week, and then divided into 3 groups of 10 each using a randomized block design; a normal diet group (ND), high-fat diet group (HD), and carnitine-supplemented high-fat diet (HDC) group (Table 1; Research Diet, New Brunswick, NJ, USA). Each group was fed its respective AIN-93 modified diet for 12 weeks (Table 1). The animals had free access to food and water and were housed in a temperature (23±1°C) and humidity (53±2%) controlled environment with a 12 hr (08:00-20:00) light-dark cycle.

Serum and tissue samples Food was withheld from the experimental animals for 12 hr before sacrificing. Blood samples were collected by orbital venipuncture and held on ice for 1 hr after which serum was separated from the blood by centrifugation at 1,000×g for 10 min and kept at -80°C until analyzed. Livers were rinsed with a phosphate buffered saline solution, wiped with a paper towel, put in a 1.5 mL eppendorf tube, labeled, and stored at -80°C until

Table 1. Composition of experimental diet

Ingredient (g)	Normal diet[1)]	High-fat diet[2)]	High-fat diet+C
Casein, lactic	200	200	200
L-Cystine	3	3	3
Corn starch	315	-	-
Maltodextrin	35	125	125
Sucrose	350	68.8	68.8
Cellulose	50	50	50
Soybean oil	25	25	25
Lard	20	245	245
Mineral mix	10	10	10
Dicalcium phosphate	13	13	13
Calcium carbonate	5.5	5.5	5.5
Potassium citrate	16.5	16.5	16.5
Vitamin mix	10	10	10
Choline bitarate	2	2	2
FD&C Yellow dye #5	0.05	-	-
FD&C Blue dye #1	-	0.05	0.05
L-Carnitine[3)]	-	-	3.87
Total	1055.05	773.85	777.72
kcal	4057	4057	4057
kcal/g	3.8	5.2	5.2

[1)]AIN-93 Modified diet with 4% fat (10% fat calorie) content.
[2)]AIN-93 Modified diet with 35% fat (60% fat calorie) content.
[3)]35% fat diet containing 0.5% L-carnitine.

assayed. The abdominal fat pads were removed, weighed, quickly frozen in liquid nitrogen, and stored at -80°C until assayed.

Analysis of lipid and leptin levels in serum Total cholesterol (TC) and triglyceride concentrations were measured for serum and liver. Triglycerides were measured using a commercial kit (Asan Pharm. Co., Seoul, Korea) by the lipase-glycerol phosphate method. High density-lipoprotein cholesterol (HDL-C) concentration was analyzed by phosphotungstic acid-Mg using a commercial kit (Asan Pharm. Co.). Serum leptin levels were determined with a ^{125}I-labeled leptin RIA commercial kit (Linco Research, St. Charles, MO, USA). ^{125}I levels were measured in a gamma scintillation counter.

Analysis of carnitine in serum and liver Livers were prepared using 29 volumes of 0.3 M-perchloride acid (PCA). Homogenates were centrifuged at 1,500×g and separated from the supernatant. Blood and tissues were prepared for carnitine analysis using the Cederblad and Lindstedt method (16). Acid-insoluble acylcarntine (AIAC) was removed using perchloric acid precipitation and centrifugation. An aliquot of the supernatant was assayed to determine nonesterified carnitine (NEC), and another aliquot was hydrolyzed with 0.5 N KOH and assayed for total acid-soluble carnitines (TCNE). Acid-soluble acylarnitine (ASAC) was determined as the difference between total acid soluble carnitine and NEC. The pellets containing AIAC were drained, washed, and hydrolyzed in 0.5 N KOH for 60 min in a hot water bath at 60°C. In each case, carnitine was assayed using carnitine acetyltransferase (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 37°C. The supernatant was passed through an ion exchange resin column. [1-^{14}C] acetyl carnitine was measured in a liquid scintillation counter.

mRNA levels in liver Total RNA was extracted with Trizol reagent. The concentration was confirmed with a spectrophotometer, and reverse transcription–polymerase chain reaction (RT-PCR) was carried out using a one-step RT-PCR kit (ABgene, NY, USA) for cDNA synthesis on an RT-PCR machine (MWG-Biotech, High Point, NC, USA). β-Actin cDNA was also prepared as a control. Upon completion of the reaction, cDNA products were visualized following 1.0% agarose gel electrophoresis. By this method we confirmed the expression of acyl-coA synthetase (ACS), carnitine palmitoyl transferase-I (CPT-I), acetyl-coA carboxylase (ACC), and acyl-coA oxidase (ACO). The relative intensity of all mRNAs was analyzed using the AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, CA, USA).

CPT-I activity in liver Hepatic mitochondria were separated by the sucrose density centrifugation method of Johnson and Lardy (17). A modified procedure of Guzman *et al.* (18) was used to estimate CPT-I activity in liver mitochondria. Each assay mixture contained a total volume of 1.0 mL: 80 mM sucrose, 1.0 mM EGTA, 70 mM imidazol, 1 μg antimycin A, 2 mg bovine serum albumin, 0.5 mM L-carnitine (0.4 mCi/mmol of L-[methyl-^{3}H] carnitine), and 40 μM palmitoyl CoA. The reactions were initiated by the addition of mitochondria or detergent extracts. The reaction was linear for up to 10 min. All reactions were performed at 37°C for 5 min. The reactions were stopped by the addition 4 mL of 1.0 M perchloric acid. The reaction mixture was centrifuged at 2,000×g for 10 min, and an aliquot of the butanol phase was transferred to a vial for counting radioactivity in a liquid scintillation counter.

Statistical analysis All values are expressed as means ± standard deviation. SAS software was used to determine significant differences between each group by ANOVA and Duncan's multiple range test at $p<0.05$ levels.

Results and Discussion

Body weight and food intake There were no significant differences in initial body weight among the groups. As shown in Table 2, final weight was significantly higher in the high-fat diet group compared with the other groups. However, carnitine supplementation prevented the increased weight gain resulting from the high-fat diet. Also, feed intake was significantly lower in the carnitine group compared to the high-fat diet groups. The energy intake and feed efficiency ratio increased the most in the HD group. The final weight of mice in the carnitine supplemented group was reduced by 14%, and feed intake was reduced by 27% compared with the HD group. In previous studies, final weight, weight gain, feed intake, and abdominal fat weight were also significantly increased by high-fat diets (19). Pigs fed L-carnitine may improve energy utilization

Table 2. Body weight and food intake of mice

	ND	HD	HDC
Initial weight (g)	17.50±0.35[1)]	17.38±0.44	17.38±0.19
Final weight (g)	25.20±1.60[c2)]	41.14±2.38[a]	35.35±3.65[b]
Weight gain (g/day)	0.13±0.06[c]	0.33±0.10[a]	0.24±0.11[b]
Feed intake (g/day)	2.60±0.16[b]	3.35±0.70	2.44±0.29[b]
Energy intake (kcal/day)	9.90±0.63[c]	17.40±3.61[a]	12.68±1.51[b]
Feed efficiency ratio[3)]	0.05±0.01[c]	0.13±0.04[a]	0.09±0.02[b]

[1)]Mean±SD of 6 mice per group.
[2)]Values with different symbols within the same row are significantly different at $p<0.05$ by Duncan's multiple range test.
[3)]Feed efficiency ratio was calculated as weight gain (g/day)/dietary intake (g/day).

Table 3. Lipid concentrations in serum

	ND	HD	HDC
HDL-C[1)] (mg/dL)	64.49±7.37[2)b3)]	98.18±8.08[a]	105.77±20.46[a]
TC (mg/dL)	130.79±16.90[b]	190.83±18.93[a]	175.66±14.15[a]
HDL-C/TC (%)	49.70±6.39[b]	51.64±4.07[b]	59.95±8.65[a]
Triglyceride (mg/dL)	142.93±41.59	157.69±36.01	151.20±25.93
LDL-C (mg/dL)	30.99±22.78[b]	66.59±18.28[a]	39.65±16.16[b]

[1)]HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol.
[2)]Mean±SD of 6 mice per group.
[3)]Values with different symbols within the same row are significantly different at $p<0.05$ by Duncan's multiple range test.

from soy oil, however pigs fed no L-carnitine may satisfy their energy requirements by increasing feed intake (20). We assume that reductions in the amounts of feed and energy consumed were responsible for the decreased weight gain. In previous research (14, 21), carnitine had no affect on feed intake. However in this study, carnitine supplementation decreased the amount of feed consumed to the level of the normal diet group. Thus, we suggest that carnitine supplementation normalizes feed intake.

Lipid profiles in serum Serum HDL-C, TC, and low-density lipoprotein cholesterol (LDL-C) were significantly higher in the high-fat diet groups (Table 3). The HDL/TC ratio was significantly higher in the HDC group than the HD group. However, serum triglyceride (TG) levels were not significantly different among the groups. High-fat diets induce lipid accumulation in the liver, and increase TG and TC concentrations in the blood (22). Birkenfeld *et al.* (23) reported that carnitine supplementation could not change blood lipids, because TG secretion from liver to blood increased while the decomposition of lipoprotein by lipoprotein lipase decreased. Although TG and TC levels were not changed by L-carnitine supplementation, the ratio of HDL/TC was higher in the carnitine supplemented group. We assume that carnitine from food intake does not improve lipid levels, and we assume that food composition has a more direct effect on blood lipid levels.

Carnitine concentrations in serum and liver Serum ASAC, AIAC, and TCNE were higher in the carnitine supplemented group compared to the high-fat diet group. The serum acyl/free carnitine ratio was significantly lower in the HD group. Liver NEC, AIAC, and TCNE were

Table 4. Carnitine concentrations in serum and liver

		ND	HD	HDC
Serum (nmol/mL)	NEC[1)]	1.01±0.17[2)b3)]	1.55±0.20[a]	1.57±0.32[a]
	ASAC	2.63±0.26[b]	2.08±0.60[b]	4.54±0.45[a]
	AIAC	0.29±0.13[b]	0.10±0.06[c]	0.46±0.09[a]
	TCNE	6.83±0.68[b]	5.12±1.06[c]	9.89±0.75[a]
	Acyl/Free	2.92±0.56[a]	1.41±0.44[b]	3.31±0.78[a]
Liver (nmol/mg wet wt.)	NEC	0.60±0.12[b]	0.27±0.02[c]	1.62±0.16[a]
	ASAC	0.76±0.22[a]	0.44±0.09[b]	0.31±0.05[b]
	AIAC	0.06±0.02[a]	0.02±0.01[b]	0.06±0.02[a]
	TCNE	1.36±0.36[b]	0.64±0.28[c]	1.97±0.46[a]
	Acyl/Free	1.38±0.18[a]	1.58±0.41[a]	0.21±0.03[b]

[1)]NEC, non-esterified carnitine; ASAC, acid-soluble acyl carnitine; AIAC, acid-insoluble acylcarnitine; TCNE, total carnitine; Acyl/Free, (ASAC+AIAC)/NEC ratio.
[2)]Mean±SD of 6 mice per group.
[3)]Values with different symbols within the same row are significantly different at $p<0.05$ by Duncan's multiple range test.

significantly higher in the HDC group than other groups. The liver acyl/free carnitine ratio was higher in the HD group than in the HDC group (Table 4). Carnitine is transported from the liver to other organs after biosynthesis in the liver from the amino acids lysine and methionine (6, 24, 25). It is very important to investigate the amount of carnitine in blood and liver to understand its physiological effects, because carnitine has a unique role in the beta-oxidation of fatty acids. Carnitine concentrations in the blood and liver increased only in the carnitine-supplemented group (26, 27). All carnitine fractions were higher in the liver except for ASAC, and the liver acyl/free ratio was lower. ASAC concentrations in blood are known to increase with increased fatty acid oxidation, and are believed to be a product of liver mitochondrial beta-oxidation. The increased carnitine fractions in the carnitine supplemented group indicate that beta-oxidation in the body was increased as well. Carnitine status in the body can be gauged by blood NEC concentration or acyl/free ratio (28). Oral carnitine supplementation can restore plasma free carnitine levels thereby making more carnitine available for tissue uptake (8). The increased carnitine levels in the carnitine-supplemented group suggest that the mice required carnitine augmentation to facilitate beta-oxidation. Also, we assume that the increased carnitine in blood and liver was caused by dietary supplementation.

Abdominal fat deposition and serum leptin levels Leptin is produced by the *ob* gene in adipose cells and is reduced by food intake and lower body weight (29). According to Lin *et al.* (30), high-fat diets significantly increase serum leptin levels, much more than a low fat diet. Moreover, a high-fat diet also increases epididymal adiposity. Almind and Kahn (31) reported that C57BL/6 mice fed a high-fat diet developed hyperleptinemia, insulin resistance, glucose intolerance, as well as increased serum leptin levels, and the authors explained these results on a genetic basis. Secretion of serum leptin was increased by white adipose tissue. Increased serum leptin compensates for the augmentation of glucose uptake in adipose tissue to prevent adiposity (32). As expected, in the current study a high-fat

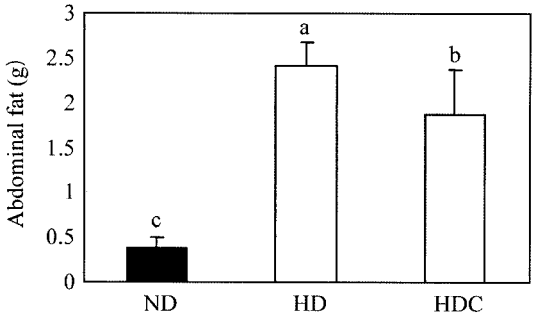

Fig. 1. Abdominal fat weight of mice. Mean±SD of 6 mice per group. Values with different symbols within the same row are significantly different at $p<0.05$ by Duncan's multiple range test.

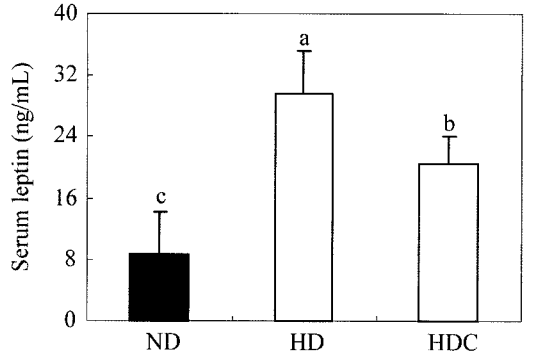

Fig. 2. Leptin concentrations in serum. Mean±SD of 6 mice per group. Values with different symbols within the same row are significantly different at $p<0.05$ by Duncan's multiple range test.

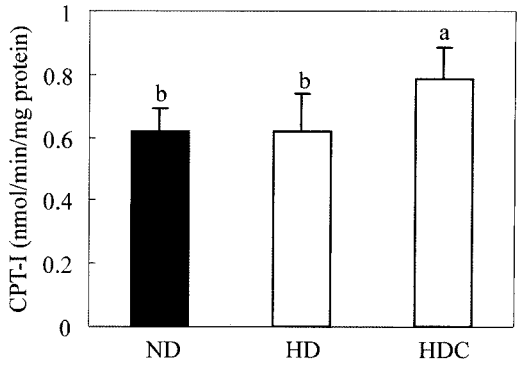

Fig. 3. CPT-1 activity in liver. Mean±SD of 6 mice per group. Values with different symbols within the same row are significantly different at $p<0.05$ by Duncan's multiple range test. CPT-1, Carnitine palmitoyl transferase-I.

diet resulted in increased abdominal fat deposits, as well as serum leptin levels. However, the weight of white adipose tissue and serum leptin levels in the carnitine-supplemented group were notably lower (Fig. 1 and 2). We suggest that carnitine prevents hyperleptinemia, insulin resistance, glucose intolerance, and furthermore prevents obesity by retarding glucose uptake.

CPT-I activity in liver There was no difference in CPT-I activity between the high-fat and the normal diet groups (Fig. 3). However, the carnitine supplemented animals had significantly higher CPT-I activity, even though they had

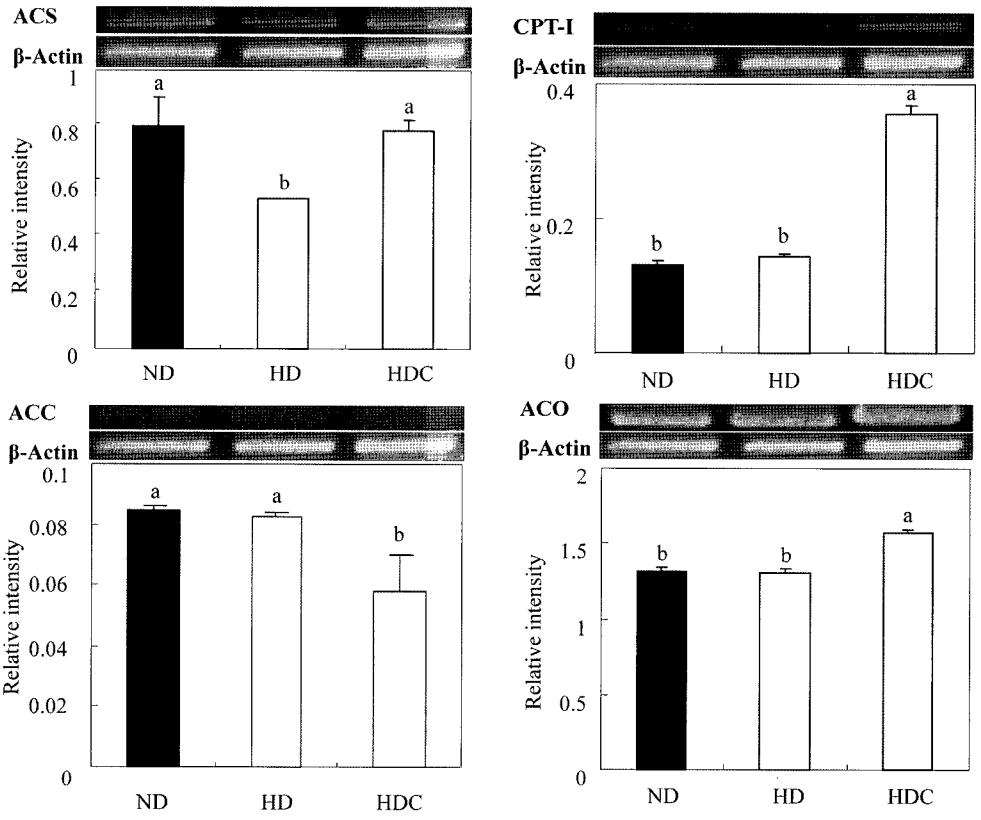

Fig. 4. mRNA expression in liver. Values with different symbols within the same row are significantly different at $p<0.05$ by Duncan's multiple range test. ACS, acyl-coenzyme A synthetase; CPT-I, carnitine palmitoyltransferase-I; ACC, acetyl-coenzyme A carboxylase; ACO, acyl-coenzyme A oxidase.

the same high-fat diet as the HD group. Geelen *et al.* (33) reported that the sensitivity of CPT-I to inhibition by malonyl-CoA was lower after the consumption of fat. Peffer *et al.* (34) reported that studies implicating CPT-I in reduced mitochondrial fatty acid flux suggested that increased CPT-I activity may promote increased beta-oxidation. We considered CPT-I activity to have increased in the carnitine supplemented group because a high-fat diet increases the amount of fatty acids transported into the mitochondria. These results suggest that carnitine supplementation increases the expenditure of fatty acids, which is augmented by a high-fat diet.

ACS, CPT-I, ACC, and ACO mRNA expression in liver Hepatic ACS, CPT-I, and ACO mRNA expression was significantly higher in the carnitine supplemented group than in the control groups. ACC mRNA expression in the liver was significantly lower in the carnitine supplemented group than the control groups (Fig. 4). ACS catalyzes the first reaction in fatty acid metabolism. ACS mRNA is abundant in liver, adipose, and heart tissue (35). CPT-I is the first step in the transport of long-chain fatty acids from the cytosol into the mitochondrial matrix for fatty acid oxidation (36). ACC, the first enzyme in the fatty acid synthesis pathway, mediates these effects on fatty acid synthesis. The rate of enzyme synthesis or degradation in the liver changed upon dietary manipulation (37). The ACO gene, which encodes the first enzyme of peroxisomal fatty acid beta-oxidation, is believed to reflect the level of peroxisomal fatty acid catabolism (38). During physiological states in which lipogenesis is occurring, ACC is activated and the associated high levels of malonyl-CoA serve to inhibit CPT-I and thereby prevent the simultaneous and futile oxidation of fatty acids by preventing their entry into the mitochondria (39). Therefore, these results suggest that dietary carnitine decreases lipogenesis, and that the decreased lipogenesis may be responsible for the reduced obesity in the carnitine supplemented mice.

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