L-Carnitine Changes the Levels of Insulin-like Growth Factors (IGFs) and IGF Binding Proteins in Streptozotocin-induced Diabetic Rat

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Summary This study investigated the effects of L-carnitine on insulin-like growth factor-I/II (IGF-I/II) and insulin-like growth factor binding proteins (IGFBPs) in streptozotocin (STZ)-induced diabetic rats. Each rat in the three L-carnitine-treated groups was injected subcutaneously with L-carnitine, 50 (D50), 100 (D100), or 200 (D200) mg/kg body weight every other day for four weeks, and animals in normal (N) and diabetic (DM) groups received saline by the same method. Diabetic rats had significantly lower carnitine concentrations in serum and liver compared with normal rats. Total carnitine concentrations were increased dose-dependently by carnitine treatment. Total IGF-I in serum from diabetic rats was increased dose-dependently by carnitine treatment, but was statistically significant only in the D200 group. The expression of liver IGF-I mRNA was lower in diabetic rats than in normal rats and increased by L-carnitine treatment. L-Carnitine treatment of diabetic rats had no effect on the levels of IGF-II in serum, liver, and kidney. Although the levels of IGF-II in serum and kidney of diabetic rats were increased in comparison with normal rats, IGF-II mRNA was not expressed in liver. Diabetic rats had markedly lower IGFBP-3 than normal rats did, and IGFBP-3 was increased by L-carnitine treatment. These results demonstrate that L-carnitine treatment of diabetic rats modulates the IGFs/IGFBPs axis. Especially noteworthy is that L-carnitine at a dose of 200 mg/kg/48 h for four weeks was able to restore serum total IGF-I in STZ-induced diabetic rats to nearly normal levels.

Key Words IGFs, IGFBPs, carnitine, diabetes

Insulin-like growth factors (IGFs) are well known to have insulin-like action and growth-promoting mitogenic effects. Insulin and IGFs, which are involved in the pathogenesis of diabetes, have structural and functional homology with each other (1). IGFs are bound to specific binding proteins (IGFBPs) as they circulate in blood. Six types of IGFBPs have been found in serum and various fluids; they control distribution of the IGFs and regulate their half-life, availability, and activity (2, 3). IGFs and IGFBPs play an important role in controlling glucose homeostasis (4), and there is evidence to support their involvement in complications related to diabetes (5). Furthermore, experimental animal and human studies both suggest differences in the IGFs/IGFBPs system between diabetic and normal states.

Carnitine (β -hydroxy- γ -trimethylaminobutyric acid) is a small water-soluble quaternary amine that plays an important role in lipid catabolism where it serves as an essential cofactor for the transport of long-chain fatty acids, as acylcarnitine esters, across the inner mitochondrial membrane (6). Recent research suggests that besides carnitine's role in the oxidation of fatty acids, it is also crucial in the regulation of carbohydrate metabolism (7). Several studies on carnitine metabolism have reported decreased plasma and liver carnitine concen-

trations in diabetic humans and experimental diabetic rats. It has been observed that carnitine administration has a hypoglycemic effect on diabetic rats (8, 9) and leads to an improvement in glucose metabolism through an insulin-sparing mechanism (10). Also, acetyl-L-carnitine at a dose of 150 mg/kg/d given for one month normalizes nerve conduction velocity in streptozotocin (STZ)-induced diabetic rats with no adverse effect (11). Therefore carnitine might be useful as an adjunct therapy in the treatment of diabetes mellitus (12).

Hudson et al. (13) recently reported that IGF-I itself is capable of significantly increasing CPT activity. Therefore in this study we evaluated the effects of L-carnitine on the levels of IGFs and IGFBPs in STZ-induced diabetic rats.

MATERIALS AND METHODS

Experimental animals. Forty male Sprague-Dawley rats weighing 205.3 ± 12.5 g were used. Diabetes was induced in 24 of the rats by a single intraperitoneal injection of STZ (45 mg/kg body weight, Sigma Chemical Co., St. Louis, USA) and was confirmed by a determination of urinary glucose excretion. Diabetic rats were then randomly divided into four groups: the control group and three experimental groups for L-carnitine treatment. Each rat in the three L-carnitine-treated groups was given carnitine at doses of 50, 100, or

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200 mg/kg body weight by subcutaneous injection every other day for four weeks, and animals in the control groups received saline by the same method. During the experimental periods (four weeks), the rats were fed an AIN-76 diet, housed in a laboratory maintained at a constant temperature ($23 \pm 2^\circ\text{C}$), and controlled in a 12-h light/dark cycle. The animals were given free access to food and water during the entire experimental period. At the end of this period, they were anesthetized following a 12-h fast. Blood samples were taken from the trunk after decapitation and centrifuged at 4°C , 3,000 rpm for 30 min, and serum was separated and stored at -70°C until assayed. Liver and kidney were removed and rinsed with a saline solution, wiped with paper towel, then homogenized with trifluoroacetic acid solution (TFA) for total IGFs and centrifuged at 4°C , 3,000 rpm for 30 min; the supernatant was separated and stored at -70°C until assayed.

Carnitine assay. Total carnitine in serum and liver extracts were determined by the radioenzymatic procedure of Cederblad and Lindstedt (14), as modified by Sachan et al. (15). In this method all samples were hydrolyzed with 0.5 mol/L KOH for 60 min in a hot water bath at 65°C . An aliquot of the neutralized supernatant was used to assay the total carnitine. In each sample, carnitine was assayed by using carnitine acetyl transferase (Sigma Chemical Co., St. Louis, MO, USA) to esterify the carnitine to a [^{14}C]acetate from [$1\text{-}^{14}\text{C}$]acetyl CoA (Amersham, Arlington Heights, IL, USA). The radioactivity of samples was determined in a Beckman LS3801 liquid scintillation counter (Beckman Instruments, Palo Alto, CA, USA).

IGF-I/II radioimmuno assay (RIA). Recombinant human IGF-I/II was iodinated to a specific radioactivity of 150–300 Ci/g, [^{125}I] by a modification of the chloramin-T method. Serum and tissue IGF-BPs were separated by the method of Lee and Henricks (16). Immunoreactive IGF-I/II was performed by the method of Lee et al. (17). Fifty μL of polyclonal IGF-I/II antibody diluted to 1:1,000 were added to 100 μL of samples/standard, then incubated for 1 h at room temperature. Next, [^{125}I]-IGFs measuring 20,000 cpm were added to the samples/standard and incubated for 18 h at 4°C . All samples were centrifuged at $3,000 \times g$ for 30 min. The supernatant was discarded, and radioactivity of the precipitate containing bound [^{125}I]-IGFs was detected in the gamma scintillation counter. All assays were performed in duplicate. Inter- and intra-assay coefficients of variation for IGFs were 8% and 10%, respectively.

IGF-I/II mRNA expression assay by quantitative competitive reverse transcription and polymerase chain reaction (QC RT-PCR). Total RNA was separated, then quantified by the method of Fichera et al. (18). Briefly, total RNA was isolated from liver samples of all animals with TRIzolTM Reagent (Gibco BRL, Basle, Switzerland), and the pellet was resuspended in DEPC water. The amount of total RNA was measured spectrophotometrically at 260 nm. RT-PCR reaction was carried out as follows: Three ng of RNA resuspended with 4.1 μL of diethylpyrocarbonate distilled water (DW) was mixed with reac-

tion mixture containing 2 μL of 5X transcription optimized buffer, 1 μL of 0.1 M DL-dithiothreitol (DTT), 1 μL of 500 μM dNTP, 1 μL of random primer (1 ng/mL), 0.4 μL of RNase inhibitor, and 0.5 μL of transcriptase to 10 μL of total volume and the incubation was prolonged for 1 h at 42°C and 10 min at 42°C . Q-PCR reaction was then performed as follows: Ten μL of each sample containing 3 ng of RT product, 1 μL standard (3.5 ng), and 6 μL of DW was diluted in 50 μL reaction buffer containing 5 μL of 10X buffer, 5 μL of 500 μM dNTP, 3 μL of MgCl_2 , 1 μL of IGF-I/II 5' primer, 1 μL of IGF-I/II 3' primer, and 0.25 μL of Taq (Qiagen, Hilden, Germany). The Q-PCR was started at 94°C . One cycle consists of 1 min at 55°C and 1 min at 72°C for IGF-I and consists of 1 min at 58°C and 1 min at 72°C for IGF-II. The number of PCR cycles was 30 for both IGF-I and IGF-II. Ten μL of PCR products were run on a 2.5% agarose gel and dyed with ethidium bromide. The mean intensity of the target bands in agarose gel was measured with a Kodak camera (Eastman Kodak Company, Rochester, NY, USA).

Western ligand blotting (WLB). Samples were electrophoresed on 12% SDS-PAGE under nonreducing conditions by using the Mini-Protein, and proteins were electroplated onto a nitrocellulose membrane. The membrane was blocked with 1% BSA and incubated for 18 h at 4°C with 200,000 cpm of [^{125}I]-IGFs in the buffer containing 1% BSA and 0.1% Tween 20. After extensive washing in cold buffer, the membrane was dried at room temperature. X-ray films were then exposed to the membranes at -70°C for 7 d by the method of Hossenlopp et al. (19).

Statistical analysis. All values are expressed as group means $\pm$ SD. The statistical significance of differences between groups was determined by Student's *t*-test and analysis of variance using the SAS version 6 (SAS Institute, Cary, NC, USA). When the *F* test indicated differences between groups, the differences were separated by using Duncan's multiple range test.

RESULTS

Food intake was significantly higher in diabetic rats than in normal rats; however, weight gain was the same in both groups (Table 1). As shown in Table 2, total carnitine levels in serum and liver were significantly lower in diabetic rats than in normal rats. L-Carnitine treatment induced a dose-dependent increase in total carnitine concentrations in serum, but not in liver. L-Carnitine treatment at the 100 mg/kg and 200 mg/kg doses increased liver total carnitine concentrations, whereas the 50 mg/kg dose had no effect. Total IGF-I levels in serum and liver were lower in diabetic rats than in normal rats and increased dose-dependently with L-carnitine treatment. Total IGF-II levels in serum and kidney were higher in diabetic rats, but L-carnitine treatment had no effect on IGF-II levels (Table 3). Liver IGF-I mRNA expression (Fig. 1) was lower ($p < 0.05$) in diabetic rats than in normal rats and was increased by L-carnitine treatment. IGF-II mRNA expression, however, was not detected in either normal or diabetic rats or in

Table 1. Food consumption, body weight gain, and food efficiency ratio.

	Control		Carnitine-treated diabetes		
	Normal	Diabetes	D50	D100	D200
Food consumption (g/d)	16.4±1.51 ^{1,*}	23.2±1.5	22.8±2.6	24.3±2.5	23.7±4.4
Weight gain (g/d)	3.1±0.4	3.0±1.0	2.4±0.8	2.8±1.5	2.3±1.2
Food efficiency ratio ²	18.7±2.0*	12.7±3.5	10.7±3.6	11.7±6.3	10.2±5.7

¹ Values are mean ($n=8$)±SD, *; values with normal rats are significantly different compared with corresponding values of diabetic control rat by Student's *t*-test at $p<0.05$. ² The food efficiency ratio was calculated as weight gain/dietary intake during the experimental period. Dose (mg/kg body weight/48 h); D50 (dose 50), D100 (dose 100), D200 (dose 200).

Table 2. Total carnitine concentration in serum and liver.

	Control		Carnitine-treated diabetes		
	Normal	Diabetes	D50	D100	D200
Serum (nm/mL)	81.9±13.6*	62.1±19.5 ^b	112.4±29.5 ^{ab}	145.7±37.4 ^{ab}	167.8±57.5 ^a
Liver (nm/g wet weight)	305.2±18.1	297.4±82.1 ^b	278.6±21.3 ^b	366.8±50.1 ^{ab}	401.9±49.1 ^a

Values are mean ($n=8$)±SD. Values with different superscripts are significantly different by ANOVA with Duncan's multiple range test at $p<0.05$. *; Values with normal rat are significantly different when compared with the corresponding values of diabetic control rat by Student's *t*-test at $p<0.05$. Dose (mg/kg body weight/48 h) D50 (dose 50), D100 (dose 100), D200 (dose 200).

Table 3. IGF-I and IGF-II concentrations in serum, liver, and kidney.

		Control		Carnitine-treated diabetes		
		Normal	Diabetes	D50	D100	D200
Serum	T-IGF-I	628±84.8*	242.8±38.8 ^b	455.6±246 ^{ab}	571.2±109.4 ^{ab}	660.8±89.6 ^a
	(ng/mL) T-IGF-II	12.53±1.2*	18.81±6.8	18.54±5.57	17.29±3.61	16.78±1.97
Liver	T-IGF-I	73.6±11.52*	41.6±17.92	49.92±10.24	56.96±11.52	57.6±18.56
	(ng/mL) T-IGF-II	18.1±3.5	25.9±7.3	28.5±8.0	22.1±4.0	21.5±2.2
Kidney	T-IGF-I	23.2±11.2	26.4±10.4	26.4±12.8	27.2±9.6	22.4±4.2
	(ng/mL) T-IGF-II	21.5±2.1*	34.9±4.9	41.6±7.5	42.5±13.8	43.2±14.9

Values are mean ($n=8$)±SD. Values with different superscripts are significantly different by ANOVA with Duncan's multiple-range test at $p<0.05$. *; Values with normal rat are significantly different compared with corresponding values of diabetic control rat by Student's *t*-test at $p<0.05$. Dose (mg/kg body weight/d); D50 (dose 50), D100 (dose 100), D200 (dose 200), T-IGF-I; total insulin-like growth factor-I, T-IGF-II; total insulin-like growth factor-II.

L-carnitine-treated diabetic rats (data not shown). The patterns of serum IGFBPs analyzed by WLB are shown in Fig. 2, and 38–42 kDa (IGFBP-3), 34 kDa (IGFBP-2), and 24 kDa (IGFBP-4) were identified. The amount of IGFBP-3 was lower ($p<0.05$) in diabetic rats than in normal rats, but it increased with L-carnitine treatment. The amount of IGFBP-2 was decreased in the L-carnitine treated group.

DISCUSSION

This study was conducted to study the effects of L-carnitine on the levels of IGFs and IGFBPs in STZ-induced diabetic rats. Several studies reported that poor glycemic control in diabetes is associated with reduced serum IGF-I levels (4, 5). The levels of total and free carnitine in serum are decreased in diabetes, and carnitine

deficiency plays an important role in diabetes-related complications (20).

Our results showed that the levels of serum total IGF-I were increased in parallel with serum total carnitine concentrations induced by subcutaneous injections of L-carnitine.

The main source of circulating IGF-I is considered to be the liver (21). Increased total IGF-I level in serum may have been due to an increase of IGF-I production in the liver. The area ratios of IGF-I per standard bands were calculated by using area values based on a densitometric analysis (Fig. 1). In this study, the levels of IGF-I and the expression of IGF-I mRNA in liver were both increased by L-carnitine treatment. These observations demonstrated that the treatment of STZ-induced diabetic rats with L-carnitine increases hepatic IGF-I

Fig. 1. IGF-I mRNA expression in liver. The radioactivity in each animal was quantified by a Bio-imaging Analyzer (BAS-2000, Fuji, Japan). The values are the means for eight animals $\pm$ SD. * p <0.05 compared to the normal rats. Lane M, marker; Lane a, normal; Lane b, diabetes; Lane c, dose 50; Lane d, dose 100; Lane e, dose 200.

Fig. 2. IGFBPs pattern in serum of normal and streptozotocin-induced diabetic rats by Western ligand blotting (WLB). After quantitative analysis of protein, pooled samples were analyzed by WLB. Ten μ L of samples were electrophoresed on 12% SDS-PAGE gel under nonreducing conditions. The proteins were transferred onto nitrocellulose filters, incubated with [125 I]-IGF-I, and exposed to X-ray film for 7 d. The radioactivity in each animal was quantified by a Bio-imaging Analyzer (BAS-2000, Fuji). The values are the means for eight animals $\pm$ SD. * p <0.05 compared to the normal rats. IGFBPs, insulin-like growth-factor binding proteins. Lane A, normal; Lane B, diabetes; Lane C, dose 50; Lane D, dose 100; Lane E, dose 200; IGFBP-3, 42 kDa; IGFBP-2, 34 kDa; IGFBP-4, 24 kDa.

mRNA expression, which results in increased IGF-I production in liver and eventually induces increased IGF-I level in serum. However, in contrast to the increased levels of IGF-I in liver, there was a decrease in IGF-I levels in kidney by treatment with L-carnitine.

A typical characteristic of the IGFs/IGFBPs axis in diabetic rats is that IGF-II levels are either increased or unchanged. In our results, IGF-II levels in serum and kidney from diabetic rats were increased in comparison with normal rats, but the IGF-II level in liver was not different between diabetic and normal rats. Also, there was no detectable IGF-II mRNA expression in either normal or diabetic rat liver. It has been reported that IGF-II expression is not continued after birth in rodents, unlike humans (22, 23). Therefore it can be assumed that the increase of IGF-II in serum from diabetic rat was not due to an increase of IGF-II production in liver. Other tissues may be involved. Recent studies have shown that the renal IGFs/IGFBPs axis is altered in diabetes, suggesting that these changes may be implicated in alterations in renal functions and morphology that accompany diabetes. Because kidney from diabetic rats had a higher IGF-II level than in normal rats, the changes of serum IGF-II level might have been due to an increase in renal synthesis. In this study, we did not determine IGF-II expression in kidney; therefore further work is necessary to determine the relationship between IGF-II increase and renal synthesis in diabetes.

The changes in IGFBPs patterns in serum were analyzed by WLB. The amount of IGFBP-3 was markedly decreased; however the amount of IGFBP-2 and IGFBP-4 were not changed in serum from STZ-induced diabetic rats in comparison with normal rats. These results were partially explained by the findings of Luo and Murphy (24), who found a significant increase in the amount of hepatic IGFBP-1 and IGFBP-2 mRNA at one month and three months after the onset of diabetes. In contrast to the increase in hepatic IGFBP-1 and IGFBP-2 mRNA was a significant decrease in hepatic IGFBP-3 mRNA and no significant difference in hepatic IGFBP-4 mRNA levels in diabetic rats. These results may suggest that changes in serum IGFBP-3 in diabetic rats were due to changes in the hepatic IGFBP-3 pattern, and these changes may be regulated at the transcriptional level. The treatment of L-carnitine resulted in a dose-dependent increase in IGFBP-3, but there were no changes in IGFBP-2 and IGFBP-4. This study is the first to demonstrate an involvement of carnitine with the IGF system. The central role of carnitine on lipid metabolism is well known, but the role of carnitine in carbohydrate metabolism is less well understood. It has been reported that L-carnitine stimulates the activity of the pyruvate dehydrogenase complex by decreasing the intramitochondrial acetyl-CoA/CoA ratio through the trapping of acetyl groups. The simultaneous reduction of acetyl-CoA levels in the cytosol further contributes to activate the glycolytic pathway (25). Recently, De Gaetano et al. (12) demonstrated that increased circulating carnitine did not alter the insulin sensitivity index or insulin/c-peptide, but glucose disposal from

plasma was significantly increased with carnitine in healthy volunteers. They suggested that carnitine might play a role in the therapy of diabetes mellitus. Our results could suggest that L-carnitine treatment of STZ-induced diabetic rats also improved glucose metabolism via modulation of the IGFs and IGFBPs levels. The esterification of long-chain fatty acids to carnitine by CPT-I is an important and possibly rate-limiting step in fatty acid oxidation (26). The increase in CPT-I activity in STZ-induced diabetic rats was expected and is an effect seen in various species when fatty acid oxidation is increased (27, 28). CPT-I activity was not changed by L-carnitine treatment at the 50 mg/kg dose, but it had a tendency to increase at the 100 mg/kg dose and was reduced at the 200 mg/kg dose (data not shown). If subsequent research does demonstrate an effect of carnitine supplementation of STZ-induced rats on CPT-I activity, that will be an important development deserving further investigation.

In conclusion, these results demonstrate that L-carnitine treatment of STZ-induced diabetic rats modulates the IGFs/IGFBPs axis. It is especially noteworthy that L-carnitine at a dose of 200 mg/kg body weight/48 h for four weeks restores serum concentrations of total IGF-I to near normal levels.

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