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Effects of carnitine palmitoyltransferase I inhibitors on hepatic hypertrophy

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Abstract

We investigated the effect of two types of carnitine palmitoyltransferase I inhibitors, ethyl 2-(6-(4-chlorophenoxy)hexyl)oxirane-2-carboxylate (etomoxir) and (*R*)-3-carboxy-*N*,*N*,*N*-trimethyl-2-{[hydroxy(tetradecyloxy)phosphinyl]oxy}-1-propanaminium hydroxide (SDZ CPI 975), on cardiac and hepatic hypertrophy in ddY mice. One-week administration of etomoxir caused cardiac and hepatic hypertrophy, 19% and 22% as a ratio to body weight, respectively. Although 4-week administration of etomoxir caused hepatic hypertrophy, there was no significant change in liver triglyceride content in the first or second week. In cultured $HepG_2$ cells, etomoxir treatment (1 week) did not cause triglyceride to accumulate. One-week administration of SDZ CPI 975 caused neither cardiac nor hepatic hypertrophy. In vitro, neither drug had selectivity for carnitine palmitoyltransferase I isozymes. These findings suggest that the hepatic hypertrophy following 1- or 2-week treatment with etomoxir is caused by mechanisms different from those responsible for triglyceride accumulation, and that inhibition of carnitine palmitoyltransferase I may not necessarily induce hepatic hypertrophy.

Keywords: Carnitine palmitoyltransferase; Etomoxir; SDZ CPI 975; Hypertrophy

1. Introduction

In patients with non-insulin-dependent diabetes mellitus, elevated free fatty acid levels contribute to the excessive fatty acid oxidation-induced production of glucose in the liver (Foley, 1992). Carnitine palmitoyltransferase I is involved in the transport of long-chain fatty acids into mitochondria and is a rate-limiting step of mitochondrial long-chain free fatty acid oxidation (McGarry and Foster, 1973; Cook et al., 1980). At least two types of carnitine palmitoyltransferase I exist, liver-type carnitine palmitoyltransferase I and muscle-type carnitine palmitoyltransferase I, on the outer membrane of mitochondria. These isoforms have different sequences of amino acids with different physical and kinetic properties (McGarry et al., 1983; Saggerson and Carpenter, 1981; Woeltje et al., 1990).

Carnitine palmitoyltransferase I inhibitors, etomoxir and SDZ CPI 975, have been developed as therapeutic agents for diabetes mellitus with hepatic glucose production induced by excessive oxidation of fatty acids. Etomoxir, an irreversible carnitine palmitoyltransferase I inhibitor, has potent hypoglycemic activity in fasted C57 BL/KsJ-db/db mice (Wolf, 1992) and patients (Selby and Sherratt, 1989; Ratheiser et al., 1991). However, the clinical development of etomoxir was discontinued, possibly because oxirane carboxylate derivatives such as etomoxir are active in the heart, as well as the liver, and are associated with cardiac hypertrophy (Bressler et al., 1989; Anderson et al., 1995). The lesser selectivity of liver-type carnitine palmitoyltransferase I and the irreversible effect of etomoxir may be involved in cardiac hypertrophy (Bressler et al., 1989). SDZ CPI 975, a reversible carnitine palmitoyltransferase I inhibitor, was developed as a liver-type carnitine palmitoyltransferase I-selective inhibitor. SDZ CPI 975 had no effect on cardiac size or function on oral administration for 26 weeks (Anderson et al., 1995). It seems to be difficult to make a rapid and early evaluation of the various compounds involved in cardiac hypertrophy.

It is likely that triglyceride accumulation is a key factor in inducing hepatic hypertrophy because consecutive intra-

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peritoneal injections of etomoxir (18 mg/kg) for 8 days caused triglyceride accumulation in liver in rats (Schmitz et al., 1995). However, there is no report with regard to hepatic hypertrophy induced by etomoxir. Additionally, it is not known whether SDZ CPI 975 causes triglyceride accumulation in the liver and hepatic hypertrophy.

In this study, we investigated whether etomoxir caused cardiac and hepatic hypertrophy in ddY mice in the short term, 1 week. We also tried to elucidate the relation between hepatic hypertrophy, triglyceride accumulation and plasma enzyme activities to monitor liver and cardiac muscle injuries.

2. Materials and methods

2.1. Materials

Ethyl 2-(6-(4-chlorophenoxy)hexyl)oxirane-2-carboxylate (etomoxir), etomoxir-CoA and (*R*)-3-carboxy-*N*,*N*,*N*-trimethyl-2-{[hydroxy(tetradecyloxy)phosphinyl]oxy}-1-propanaminium hydroxide (SDZ CPI 975) were synthesized at the Central Research Laboratories of Nissan Chemical Industries (Chiba, Japan). The compounds were dissolved or suspended in 0.5% methyl cellulose (SM-400; Shinetsu Chemical, Tokyo, Japan). Streptozotocin, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), bovine serum albumin (essential fatty acid-free) and L-carnitine were purchased from Sigma (St. Louis, MO, USA). Palmitoyl-CoA (free acid) was purchased from Funakoshi (Tokyo, Japan). Ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) was purchased from Wako (Osaka, Japan).

2.2. In vitro study

2.2.1. Preparation of intact mitochondria

After being fasted for 16 h, 6-week-old male ddY mice (SLC, Shizuoka, Japan) were injected intraperitoneally with a bolus of 150 mg/kg of streptozotocin dissolved in 3 mM citrate buffer (pH 4.5). After 2–4 weeks, mice with >300 mg/dl glucose levels in plasma were used for preparation of mitochondria. Intact mitochondria were isolated by a modification of the procedure of McGarry et al. (1978). The streptozotocinized mice were killed, and the liver or ventricle of the heart was isolated. These tissues were minced with scissors and homogenized in 0.25 M sucrose (10 g tissue/25 ml sucrose solution). The homogenate was centrifuged for 15 min at $600\times g$ to remove the nuclear fraction, and the supernatant was centrifuged for 15 min at $7700\times g$. The pellet was then suspended to the original volume in 0.25 M sucrose and centrifuged for 15 min at $7700\times g$. The pellet was then resuspended in 150 mM KCl, 5 mM Tris–HCl (pH 7.2) and stored at -80°C before assays of carnitine palmitoyltransferase I activity. All the procedures were conducted at 4°C.

2.2.2. Assay of carnitine palmitoyltransferase I activity

Carnitine palmitoyltransferase I activity was assayed by the modified method of Clarke and Bieber (1981). Assays were carried out at 25°C. The 1-ml reaction mixture included 50 mM Tris–HCl (pH 7.4), 0.1 mM palmitoyl-CoA, 0.1 mM DTNB, 5 mM $MgCl_2$, 0.5 mM EDTA, 12.5 mg of bovine serum albumin, liver or heart mitochondria suspension (0.12–0.25 mg protein) and dimethyl sulfoxide (DMSO) containing a test compound. The final concentration of DMSO in the reaction mixture did not exceed 0.5%. The reactions were initiated by the addition of L-carnitine (final concentration = 1.6 or 16 mM), and then the changes in absorbance at 412 nm for 1 min were determined.

2.3. In vivo study

Male 6-week-old ddY mice were used. The mice were divided into three groups: (1) 0.5% methyl cellulose (control group; $n=5$); (2) 100 mg/kg of etomoxir group ($n=5$); and (3) 100 mg/kg of SDZ CPI 975 group ($n=6$). Each compound was administered p.o. (0.1 ml/10 g body weight) once a day for a week. In another experiment, 100 mg/kg of etomoxir was administered once a day for 4 weeks, and three or four mice were killed every week. Food and water were given ad libitum. Twenty-four hours after the final administration, the mice were killed to collect blood samples, and to isolate liver and heart tissues. The blood samples from the abdominal artery were used for analysis of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, creatinine phosphokinase and lactate dehydrogenase. Enzyme activity was determined with the appropriate test kit (Wako). The liver and heart weight were determined gravimetrically. The degree of hepatic and cardiac hypertrophy was expressed as the ratio of the weight of each tissue to 100 g of body weight. A part of each liver was homogenized in phosphate-buffered saline (PBS) (10 g of tissue/25 ml buffer), and total lipids were extracted with a chloroform–methanol mixture (C/M; 2:1, v/v). The amount of triglyceride extracted was determined with a test kit (Kyowa Medex, Tokyo, Japan).

2.4. Trigliceride content of cultured $HepG_2$

The $HepG_2$ cells, human hepatoma cells, were provided by Prof. Saito, Chiba University, Chiba, Japan. The cells were seeded on a 12-well plastic culture plate and cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Bio Whittaker, Walkersville, Maryland, USA) under 5% CO_2 in air at 37°C. After the cells had reached sub-confluence, various concentrations of etomoxir or SDZ CPI 975 were added. The compounds were dissolved in DMSO. The final concentration of DMSO in the culture medium was not in excess of 0.1%. The culture medium was changed every second day. After 1 week, the cells

were washed with cold PBS twice. They were scraped off with a rubber policeman into cold PBS, and then transferred to tubes (1.5 ml vol). After the cells were sonicated, the total lipids were extracted with a chloroform–methanol mixture (C/M; 2:1, v/v). The triglyceride contents were measured with the test kit described in Experimental Procedure 2.3. The proteins were quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA).

2.5. Statistical analyses

All results are expressed as the means ± S.E.M. Statistical analysis was done using Super Anova version 1.11 (Abacus Concepts, Berkeley, CA, USA) on a Macintosh computer. Student's *t*-test was applied to the data in Fig. 3 and Table 2. Dunnett's multiple test was applied to the data in Table 1. Probability values less than 5% were considered significant.

3. Results

3.1. Effects of SDZ CPI 975 and etomoxir-CoA on carnitine palmitoyltransferase I activity of liver and heart mitochondria

We investigated the effects of SDZ CPI 975 and etomoxir-CoA, the active form of etomoxir, on the carnitine palmitoyltransferase I activity of liver and heart mitochondria (Fig. 1). Both drugs inhibited the carinitine palmitoyltransferase I activity of the preparations in a concentration-dependent manner. The inhibitory effects of SDZ CPI 975 did not differ between the two preparations. The IC_{50} values of SDZ CPI 975 were 33.8 and 46.0 μM for liver and heart, respectively. Similarly, etomoxir-CoA had no selectivity for the carnitine palmitoyltransferase I isozymes. The IC_{50} values of etomoxir-CoA were 9.7 and 14.1 μM for liver and heart, respectively.

Furthermore, we investigated the effect of carnitine on the inhibitory action of SDZ CPI 975 and etomoxir-CoA on carnitine palmitoyltransferase I activity of liver (Fig. 2). The effect of both doses (30 and 100 μM) of SDZ CPI 975 on carnitine palmitoyltransferase was greatly reduced by 16 mM of carnitine. The effect of 30 μM of SDZ CPI 975 was completely lost. There was no difference in the extent of inhibition by either dose of SDZ CPI 975. On the other hand, the effect of etomoxir-CoA was not affected by 16 mM carnitine as were those of both doses of SDZ CPI 975. A total of 16 mM of carnitine reduced by only about 20% the carnitine palmitoyltransferase inhibition by 100 μM of etomoxir-CoA.

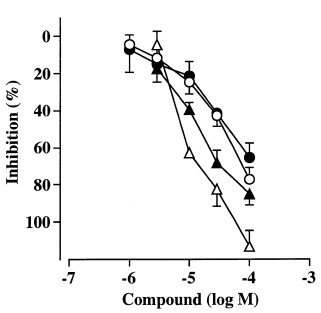

Fig. 1. Effect of SDZ CPI 975 (circle) and etomoxir-CoA (triangle) on carnitine palmitoyltransferase I activity of liver (open symbols) and heart (closed symbols) mitochondria. Each point indicates the mean ± S.E.M. for three or four separate experiments.

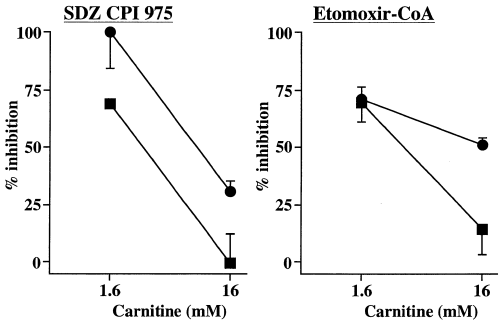

Fig. 2. Effect of carnitine on carnitine palmitoyltransferase I inhibitory action of etomoxir-CoA and SDZ CPI 975. Each point indicates the mean ± S.E.M. for three separate experiments. Square indicates 30 μM for each compound. Circle indicates 100 μM of each compounds.

3.2. Effect of carnitine palmitoyltransferase I inhibitors on cardiac and hepatic hypertrophy, and triglyceride content of liver and plasma in mice

We investigated the effects of carnitine palmitoyltransferase I inhibitors on the ratio of heart and liver weight to 100 g body weight of mice to assess cardiac and hepatic hypertrophy (Table 1). One week of treatment with etomoxir (100 mg/kg, p.o.) resulted in a significant increase (15%) of the net heart weight and (19%) of the ratio of heart weight to 100 g body weight, compared with the control group. The net liver weight and the ratio to 100 g body weight in mice treated with etomoxir also increased significantly, by 21% and 22%, respectively, compared with the control group. On the other hand, 1 week of treatment with SDZ CPI 975 did not result in a significant change of heart or liver weight. Neither etomoxir nor SDZ CPI 975 had a significant effect on body weight or triglyceride content of liver and plasma, compared with the control group.

We investigated the effects of the two carnitine palmitoyltransferase I inhibitors on plasma glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, creatinine phosphokinase and lactate dehydrogenase activities in mice. Etomoxir had no effect on these four enzyme activities. Although SDZ CPI 975 only tended to decrease the creatinine phosphokinase activity, compared with that of the

Table 1
Effects of etomoxir (100 mg/kg, p.o.) or SDZ CPI 975 (100 mg/kg, p.o.) for 1 week on cardiac and hepatic hypertrophy, and TG content of liver and plasma of ddY mice
Each value is the mean ± S.E.M.

Condition dose (mg/kg/day)	Control ($n = 5$)	Etomoxir ($n = 5$) 100	SDZ CPI 975 ($n = 6$) 100
Body weight (g)	34.4 ± 0.8	34.2 ± 0.7	34.2 ± 0.9
Heart weight (g)	0.141 ± 0.004	0.166 ± 0.003[a]	0.141 ± 0.002
Heart weight/100 g body weight	0.409 ± 0.005	0.487 ± 0.011[a]	0.412 ± 0.005
Liver weight (g)	1.857 ± 0.042	2.249 ± 0.063[a]	1.765 ± 0.055
Liver weight/100 g body weight	5.41 ± 0.15	6.58 ± 0.09[a]	5.16 ± 0.10
TG content			
Liver (μg/liver)	4369 ± 278	3412 ± 472	4531 ± 321
Plasma (μg/ml)	68.7 ± 8.4	64.8 ± 11.5	72.0 ± 8.8

[a] $P < 0.05$, significantly different from control mice (Dunnett's multiple test).

control (78.4 ± 7.8 vs. 118.2 ± 26.9 IU/l, respectively), the difference was not significant.

3.3. Effect of the 4-week treatment with etomoxir on hepatic and cardiac hypertrophy, and triglyceride content of liver in mice

To confirm the relation between hepatic hypertrophy and triglyceride content in liver, we investigated the effect of etomoxir on hepatic hypertrophy in mice. We confirmed that 1 week after the treatment with etomoxir, liver weight was significantly increased. The liver was significantly heavier in the etomoxir group than in the control group from the first to fourth week (Fig. 3). However, the hypertrophy in the first and second week was not accompanied by a significant triglyceride accumulation in liver (Table 2). Etomoxir significantly increased the triglyceride content of liver in the third and fourth week, compared with the control.

We observed significant cardiac hypertrophy in the third and fourth week after etomoxir treatment (Fig. 3). Although heart weight/100 g body weight in the first and second week tended to be greater in the etomoxir than in the control group, the differences were not significant ($P = 0.2138$ and 0.1248, respectively).

We investigated the effect of etomoxir on glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, creatinine phosphokinase and lactate dehydrogenase activities in plasma of mice. Etomoxir had no effect on glutamic oxaloacetic transaminase, glutamic pyruvic transaminase or creatinine phosphokinase activities from the first to fourth week. Consecutive oral administration of etomoxir significantly reduced the lactate dehydrogenase activity in the first week, compared with the control (253.5 ± 10.7 vs. 358.2 ± 34.8, respectively). Although lactate dehydrogenase activity in the second, third and fourth week was lower than those of the control group, the differences were not significant.

3.4. Effects of etomoxir and SDZ CPI 975 on triglyceride content of cultured $HepG_2$ cells

To investigate the direct effects of carnitine palmitoyltransferase I inhibitors on hepatic cells, we investigated the effects of etomoxir and SDZ CPI 975 on the triglyceride content of $HepG_2$ cells (Fig. 4). Neither etomoxir nor SDZ CPI 975 produced significant changes in the amount of triglyceride in the cells at 1 week, although treatment at 30

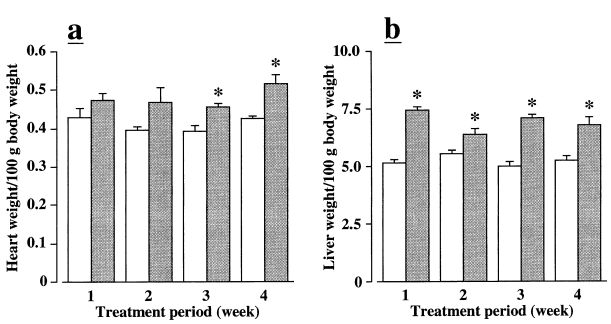

Fig. 3. Effects of etomoxir (100 mg/kg, p.o.; closed column) given for 4 weeks on cardiac (a) and hepatic hypertrophy (b) of ddY mice. The means ± S.E.M from three animals are shown except for etomoxir group (four animals) in fourth week. * $P < 0.05$ (Student's *t*-test), significantly different from control mice (open column).

Table 2
Effects of etomoxir (100 mg/kg, p.o.) for 4 weeks on TG content of liver of ddY mice
Each value is the mean ± S.E.M.
Groups are the same as those described in Fig. 3.

	TG content (μg/liver)			
	1st week	2nd week	3rd week	4th week
Control	2777 ± 231	3913 ± 613	4032 ± 86	3792 ± 584
Etomoxir	3300 ± 613	4573 ± 986	7069 ± 752[a]	6193 ± 469[a]

[a] $P < 0.05$, significantly different from control mice (Student's *t*-test).

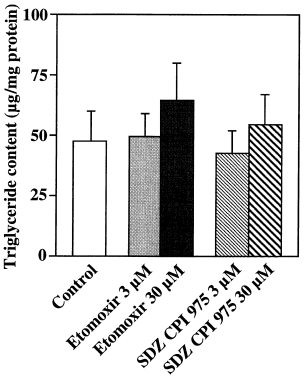

Fig. 4. Effects of 1-week treatment of etomoxir or SDZ CPI 975 on triglyceride content of $HepG_2$ cells. Each column represents the mean ± S.E.M. for four separate experiments.

μM with both drugs tended to increase the cell content of triglyceride.

4. Discussion

We showed that a 1-week treatment with etomoxir caused hepatic hypertrophy in mice. SDZ CPI 975, on the other hand, did not cause hepatic hypertrophy. SDZ CPI 975 was not a selective inhibitor for either liver or muscle type carnitine palmitoyltransferase I (Fig. 1). These results support the idea that the differences between etomoxir and SDZ CPI 975 are due to the inhibitory pattern, i.e., irreversible and reversible. It may be that etomoxir affects the cumulative effect by irreversible inhibition (Wolf., 1990) in liver. We showed that etomoxir-CoA was differrent from SDZ CPI 975 as regards reactivity for carnitine at least (Fig. 2), although we could not show clearly whether etomoxir has an irreversible inhibition profile. Intraperitoneal injection of etomoxir (18 mg/kg) for 8 days results in a significant increase in the liver content of triglyceride, 43%, in normal rats (Schmitz et al., 1995). However, we found that a 1-week treatment with etomoxir (100 mg/kg, p.o.) caused hepatic hypertrophy without triglyceride accumulation (Table 1). This effect of etomoxir was reproducible (Fig. 3). The increase in triglyceride in the third and fourth week of treatment with etomoxir was consistent with results of Schmitz et al.'s study (Table 2). These findings were also consistent with the results of our $HepG_2$ study in which a 1-week treatment with etomoxir did not produce a significant increase in the cell content of triglyceride. The present results suggest that hepatic hypertrophy at the first and second weeks of treatment with etomoxir is caused by mechanisms different from those causing triglyceride accumulation.

Peroxisomal proliferators are well known to induce hepatic hyperplasia and hypertrophy in rodents, by increasing the number of peroxisomes in their hepatocytes (Bentley et al., 1993). This proliferation is accompanied by replicative DNA synthesis and liver growth (Srinivasan et al., 1990). Peroxisomes of hepatocytes were increased in number by etomoxir and 2-tetradecylglycidic acid, carnitine palmitoyltransferase I inhibitors of oxirane analogues (Skorin et al., 1992). Etomoxir and 2-tetradecylglycidic acid in the presence of oleic acid induced peroxisomal β-oxidation in primary hepatocyte cultures. The exposure of cultured hepatocytes to 2-tetradecylglycidic acid also resulted in a marked inhibition of triglyceride synthesis (Kaikaus et al., 1993). On the other hand, 2-tetradecylglycidic acid increased the incorporation of oleate into triglyceride in perfused rat liver. These differences may due to the experimental conditions, i.e., hepatocyte culture vs. perfused liver. However, results of the hepatocyte study, in addition to our $HepG_2$ study, may also reflect the present finding that early hepatic hypertrophy was induced by etomoxir without triglyceride accumulation.

It is of interest to find whether inhibition of β-oxidation is related to peroxisome proliferation. It is likely that attenuation of β-oxidation by carnitine palmitoyltransferase I inhibition in mitochondria induced a compensatory proliferation of peroxisomes, and then caused hepatic hypertrophy. In rats, a dose of 100 mg/kg of SDZ CPI 975, administered orally, is 25-fold ED_{50} for inhibition of hepatic ketogenesis, i.e., attenuation of β-oxidation, in 18-h fasted animals (Anderson et al., 1995). Our data indicate that 100 mg/kg of SDZ CPI 975 given orally for 7 days did not cause peroxisome proliferation in mice in spite of its inhibitory action on carnitine palmitoyltransferase I. There is no report that peroxisomes proliferate in response to non-oxirane derivatives, i.e., SDZ CPI 975-like compounds. Thus, the attenuation of β-oxidation accompanied by carnitine palmitoyltransferase I inhibition does not necessarily induce peroxisome proliferation.

We found that a 1-week-only oral administration of etomoxir significantly induced cardiac hypertrophy. This is the first report of differences in the effects of two types of carnitine palmitoyltransferase I inhibitors on cardiac and hepatic hypertrophy in the short term, i.e., 1 week. On etomoxir treatment for 4 weeks, heart weight/100 g body weight at the first and second week tended to be greater in the etomoxir than in the control group, the differences not being significant, however (Fig. 3). The discrepancy between the significance for similar measurements reported in Table 1 and Fig. 3 may result from the difference of the group size in the two experiments. Twenty-six weeks of oral administration of SDZ CPI 975 (100 mg/kg/day) in rats does not cause cardiac hypertrophy, whereas etomoxir (12.5 mg/kg/day, p.o.) increases cardiac left ventricular mass by 10–15% (Anderson et al., 1995). There are three reasons for the absence of cardiac hypertrophy: (1) SDZ CPI 975 is a reversible inhibitor (Anderson et al., 1995); (2) SDZ CPI 975 is preferentially distributed to liver (Deems et al., 1998); (3) the inhibitory effect of SDZ CPI 975 is slightly more potent on liver-type carnitine palmi-

toyltransferase I than on muscle-type carnitine palmitoyltransferase I (Mann et al., 1995). In our in vitro study, however, we showed that the inhibitory effect of SDZ CPI 975 on carnitine palmitoyltransferase I was almost as potent on muscle-type carnitine palmitoyltransferase I as on liver-type carnitine palmitoyltransferase I. Because etomoxir-induced cardiac hypertrophy is caused by the inhibition of fatty acid oxidation in heart muscle at the level of carnitine palmitoyltransferase I (Bressler et al., 1989), SDZ CPI 975 administered orally does not affect heart muscle. Recently, it was reported that 2-tetradecylglycidic acid (10 mg/kg/day) treatment for 7 days in rats induced cardiac hypertrophy and an about twofold increase in plasma angiotensin II compared with the control group (Wolkowicz et al., 1999). The authors speculate that a carnitine palmitoyltransferase I inhibitor of oxirane analogs induces cardiac hypertrophy by activating the angiotensin II type 1 (AT_1) receptor, because the cardiac hypertrophy and plasma angiotensin II level were decreased by simultaneous administration of losartan, an angiotensin AT_1 receptor antagonist. Our results suggest that the non-oxirane derivative, SDZ CPI 975, does not induce cardiac hypertrophy due to activation of angiotensin AT_1 receptor.

In conclusion, we showed that a 1-week-only treatment with etomoxir caused cardiac and hepatic hypertrophy in normal mice. In contrast, SDZ CPI 975 induced neither of these hypertrophies. The differences did not depend on the selectivity for carnitine palmitoyltransferase I isozyme in vitro. Four weeks of treatment with etomoxir showed that the early hepatic hypertrophy was not accompanied by triglyceride accumulation in liver. This observation was confirmed by the findings in $HepG_2$ cells. The present results indicate that etomoxir causes hepatic hypertrophy in advance of triglyceride accumulation. The results suggest that inhibition of carnitine palmitoyltransferase I in short period may not necessarily induce cardiac and hepatic hypertrophy.

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