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Antiobesity and Antidiabetic Effects of CL316,243, a Specific β3-adrenoceptor Agonist, in OLETF Rats

Yutaka MORI[1], Yoshiki TOKUTAKE[2], Koichi HAYAKAWA[2], Akane MATSUZAWA[2], Tatsuya NAGASAWA[2], Rie NAKANO[2], Junji KURODA[2], Satoshi AKAHANE[2], and Naoko TAJIMA[3]

[1]*Department of Internal Medicine, National Higashi Utsunomiya Hospital*
[2]*Department of Discovery Research I, Kissei Pharmaceutical Co., Ltd.*
[3]*Division of Diabetes and Endocrinology, Department of Internal Medicine, The Jikei University School of Medicine*

ABSTRACT

The antiobesity and antidiabetic effects of CL316,243 (CL), a specific β3-adrenoceptor agonist, were studied in obese, spontaneously type 2 diabetic Otsuka Long-Evans Tokushima Fatty rats. In an experiment designed to prevent obesity and diabetes, 10-week-old rats with rapidly enlarging adipocytes were given CL for 20 days. Treatment with CL did not affect the amount of food intake but significantly suppressed body weight gain and body fat accumulation ; decreased plasma levels of leptin, triglyceride, and insulin ; and improved glucose intolerance, as determined with oral glucose tolerance testing. In a therapeutic experiment, 26-week-old obese rats with hypertrophic adipocytes were given CL for 20 days. Treatment with CL significantly reduced body weight and fat volume but also reduced food intake ; CL also decreased levels of leptin, triglycerides, and free fatty acids but did not improve glucose tolerance. Cellularity analysis showed that CL reduced the size of adipocytes in both the preventive and therapeutic experiments. In the preventive experiment, CL significantly increased rectal temperatures, increased expression of the uncoupling protein 1 (UCP1) gene in brown adipose tissue 2.6-fold, and increased expression of ectopic UCP1 gene in white adipose tissue 2-fold compared with levels in controls. In the therapeutic experiment CL also increased ectopic UCP1 expression in white adipose tissue 2-fold. In conclusion, CL suppressed the increase in fat accumulation in 10-week-old rats with rapidly enlarging adipocytes and reduced accumulated fat in 26-week-old rats with hypertrophic adipocytes, possibly by accelerating lipolysis via adipocyte β3-adrenoceptors and energy consumption mechanisms via UCP1.

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Key words : β3-adrenoceptor agonist, uncoupling protein 1, brown adipose tissue, white adipose tissue, Otsuka Long-Evans Tokushima Fatty rats

INTRODUCTION

Because β3-adrenoceptor agonists[1,2], effective for treating obese patients with type 2 diabetes, also promote lipolysis in white adipocytes, they might be used to treat type 2 diabetes as well as obesity[3]. To investigate the effects of specific β3-adrenoceptor agonists on adipocytes, we studied the effects of CL316,243 (CL)[4-6], which has high specificity for rodents, in obese, spontaneously type 2 diabetic Otsu-

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森　豊，徳武　巧記，早川　晃一，松澤亜可根，長澤　達也，中野　理恵，黒田　淳二，赤羽　敏，田嶼　尚子
Mailing address : Yutaka MORI, Department of Internal Medicine, National Higashi-Utsunomiya Hospital, 2160 Shimookamoto, Kawachi-machi, Kawachi-gun, Tochigi 329-1193, Japan.
E-mail : moriyutakajp@yahoo.co.jp

ka Long-Evans Tokushima Fatty (OLETF) rats[7]. Although the effects of subcutaneously administered CL have been studied in OLETF rats[6], in the present study we examined the effects of CL more precisely by dividing the experiment into an early phase (preventing setting) and a late phase (therapeutic setting). Moreover, the CL was orally administered in food to reduce the stress to the rats.

MATERIALS AND METHODS

Specific β3-adrenoceptor agonist

CL (structure: (disodium(R, R)-5{[2-(3-chlorophenyl)-2-hydroxyethyl-aminopropyl}-1, 3-benzodioxole-2, 2-dicarboxylate)) was synthesized by Kissei Pharmaceutical Co., Ltd. (Matsumoto, Japan).

Experimental animals

Four-week-old male OLETF rats (Tokushima Research Institute, Otsuka Pharmaceutical Co., Tokushima) were raised until the start of drug therapy at the age of 10 weeks or 26 weeks. The rats were housed in individual cages with a 12-hour day/night cycle at room temperature and given free access to tap water and standard rat feed (CE-2; CLEA Japan, Inc., Tokyo). All experiments were performed in accordance with the National Institutes of Health (United States) Guidelines for the Use of Experimental Animals.

Experimental protocol

This study comprised a preventive experiment and a therapeutic experiment. In the preventive experiment 10-week-old rats received either standard chow (control group, $n=6$) or chow containing 30 ppm CL (experimental group, $n=6$) for 20 days. In the therapeutic experiment 26-week-old rats received either standard chow (control group, $n=6$) or chow containing CL 50 ppm CL (experiment group, $n=6$) for 20 days. The CL dose in each setting was 3 mg/kg/day. The rats were individually housed in plastic cages. The amount of feed was measured every day, and the rats were weighed once every 4 days. On day 20 of drug therapy, rectal temperatures were measured with a digital thermometer (TD-300, Shibaura Electronics Co., Ltd., Saitama).

Biochemical tests

In both experiments, samples of blood were drawn from the orbital venous plexus under nonfasting conditions, before the start of treatment on day 21 of drug therapy (24 hours after the final drug administration). After 16 hours of fasting, the rats underwent oral glucose tolerance testing (OGTT) with a glucose load of 2 g/kg. Blood was drawn at baseline and 30, 60, 120, and 180 minutes after glucose loading. Blood levels of glucose, plasma triglycerides (TG), free fatty acid (FFA), and total cholesterol (TC) were measured enzymatically, and insulin and leptin levels were measured with enzyme immunoassay.

Weight of adipose tissue and tissue TG content

After both experiments were completed, the rats were killed under nonfasting conditions. The liver, mesenteric fat, and left-sided retroperitoneal fat, epididymal fat, and abdominal subcutaneous fat were removed, and the total tissue weight was measured. The extent of abdominal subcutaneous adipose tissue to be excised was determined with the method of Krotkiewski and Björntorp[8]. The tissues of the liver and the paravertebral muscles were homogenized, after which lipids were extracted with the method of Folch et al.[9]. The amount of TG present was then measured, and tissue TG content (mg/g tissue weight) was calculated. The liver, gastrocnemius, interscapular brown adipose tissue (BAT), and retroperitoneal white adipose tissue (WAT) were frozen with liquid nitrogen and preserved at −80°C.

Histologic examination

Retroperitoneal and subcutaneous WATs were fixed in 10% formalin and embedded in paraffin. Tissues sections 2.5 μm thick were cut, then stained with hematoxylin and eosin. To study the size of WAT cells, the area of 1,000 cells per specimen was measured with an image analyzer (Luzex 3, Nikon,

Tokyo).

Measurement of UCP mRNA expression

Total RNA was separated from frozen tissues (liver, gastrocnemius, interscapular BAT, and retroperitoneal WAT) with an RNA extraction liquid (TRIzol, Gibco-BRL, Gaithersburg, MD, USA), and the relative levels of mRNA expression were determined with a quantitative real-time polymerase chain reaction (PCR) via a TaqMan analysis (Applied Biosystems, Foster City, CA, USA) with specific primers and probes. Oligonucleotide sequences of gene-specific primers and probes for TaqMan analysis of rat uncoupling protein (UCP)1, UCP2 and UCP3 mRNAs were as follow: UCP1: forward (sense) 5′-CGGCA G-CCTTTTTCAAAGG-3′, reverse (antisense) 5′-CCTTTCTCCAAGTCGCCTATGT-3′ fluorogenic probe FAM-5′-TTGCGCCTTCTTTTCTGC-GACTCG-3′-TAMRA; UCP2: forward (sense) 5′-CCCACTGCTTCAGTTTTTCTCC-3′, reverse (antisense) 5′-TGAGATC-TGCAATACAGGCTGC-3′, fluorogenic probe FAM-5′-TTGGTTTCAAGGC-CACCGATGT-GC-3′-TAMRA; and UCP3: forward (sense) 5′-GGCGCTTTTGACTCAAGGAT-3′, reverse (antisense) 5′-ACAATCAAAGTCCTCAGC-CACA-3′, fluorogenic probe FAM-5′-TGAAGGC-GACCGCAGTTGGTTG-3′-TAMRA. The PCR conditions were 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 120 seconds.

Statistical analysis

All numerical values are expressed as means± standard error of mean (SEM). Student's t-test was used to test for significant differences between the CL-treated groups and control groups. Differences with $p<0.05$ were considered statistically significant.

Results

Body weight changes, volume of feed taken, and rectal temperature

In the preventive setting, body weight increased in the control group throughout the observation period. In contrast, increases in body weight were suppressed in the CL group after day 12, with the posttreatment weight significantly lower (415.7±5.7 g) than that of the control group (446.0±11.0 g, $p<0.05$, Fig. 1A). However, feed intake did not differ significantly between the two groups at any time. In the therapeutic setting, body weight was unchanged in the control group from 26 weeks onward, but body weights in the CL group were significantly lower than those in the control group from day 8 onward (Fig. 1B).

Food intake was 20.8±2.4 and 13.3±1.0 g/rat/day in the control and CL groups, respectively, at the start

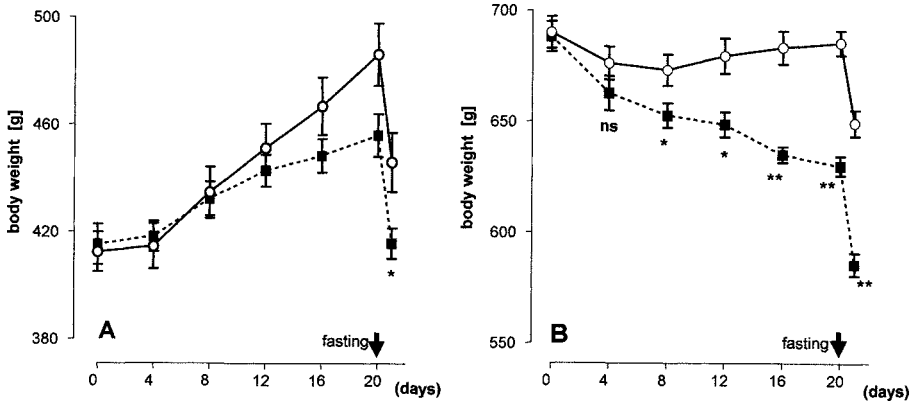

Fig. 1. Effect of CL on body weight of OLETF rats.
A: Changes in body weight with CL administration from 10 to 13 weeks after birth
B: Changes in body weight with CL administration from 26 to 29 weeks after birth ○ control group, ■ CL group (CL dose: 3 mg/kg).
$^{*}p<0.05$, $^{**}p<0.01$

of the experiment and was 25.4±0.6 and 22.6±0.7 g/rat/day in the control and CL groups, respectively, on the 20th day. These data showed that a decrease in food intake for the first week might affect body weight in the CL group, but for the subsequent 2 weeks food intake did not differ significantly between

Table 1. Biochemical test results and tissue TG content

	Preventive experiment		Therapeutic experiment	
	Control	CL316,243	Control	CL316,243
Glucose (mg/dl)	137.4±7.1	125.9±6.9	167.8±4.5	150.3±4.7*
Insulin (ng/ml)	4.28±0.57	2.38±0.17**	6.05±0.37	2.93±0.29***
Leptin (ng/ml)	10.49±0.96	4.84±0.41***	22.3±2.4	9.4±0.6***
TG (mg/dl)	246.8±32.9	140.2±13.0*	437.6±70.0	154.4±25.4**
TC (mg/dl)	110.1±5.7	99.4±1.7	197.1±15.8	149.4±2.5*
FFA (mEq/l)	0.647±0.037	0.492±0.059	0.761±0.058	0.394±0.055**
TG in liver (mg/g tissue)	35.9±1.7	32.0±3.7	73.1±4.7	84.3±5.7
TG in muscle (mg/g tissue)	6.64±1.54	3.65±0.62	14.9±3.30	9.15±3.62

*$p<0.05$, **$p<0.01$, ***$p<0.001$ versus Control

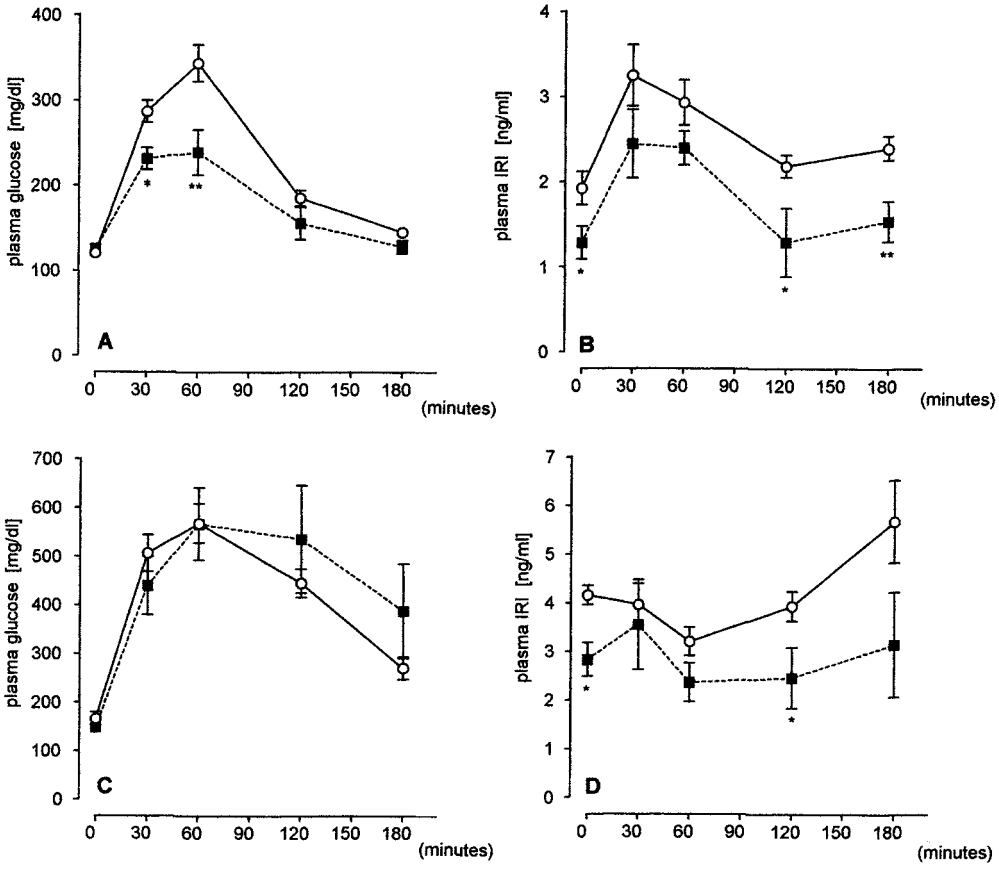

Fig. 2. Effect of CL on glucose tolerance and insulin secretion in OLETF rats after glucose loading.
Plasma glucose levels (A) and levels of immunoreactive insulin (IRI) (B) with CL administration from 10 to 13 after birth in the preventive setting. Plasma glucose levels (C) and IRI levels (D) seen with CL administration from 26 to 29 weeks after birth to in the therapeutic setting.
○ control group, ■ CL group (CL dose: 3 mg/kg).
*$p<0.05$, **$p<0.01$

the control and CL groups (data not shown). In the preventive setting, the rectal temperature on day 20 was significantly ($p<0.05$) higher in the CL group (36.80±0.19°C) than in the control group (35.98±0.16°C). However, in the therapeutic setting, rectal temperatures did not differ between the CL group (37.9±0.2°C) and the control group (37.7±0.2°C).

Biochemical tests and OGTT

In the preventive setting, plasma levels of insulin, leptin, and TG were significantly lower in the CL group than in the control group (Table 1). In the therapeutic setting, plasma levels of glucose, insulin, leptin, TG, TC, and FFA were significantly lower in the CL group than in the control group (Table 1). In the preventive setting, plasma glucose levels were significantly lower in the CL group than in the control group at 30 and 60 minutes after glucose loading, and insulin levels were also significantly lower in the CL group than in the control group at baseline, 120 and 180 minutes after glucose loading (Fig. 2A and B). In the therapeutic setting blood glucose levels at OGTT did not differ significantly between the CL group and the control group, but insulin levels were significantly lower in the CL group than in the control group at baseline and 120 minutes after glucose loading (Fig. 2C and D).

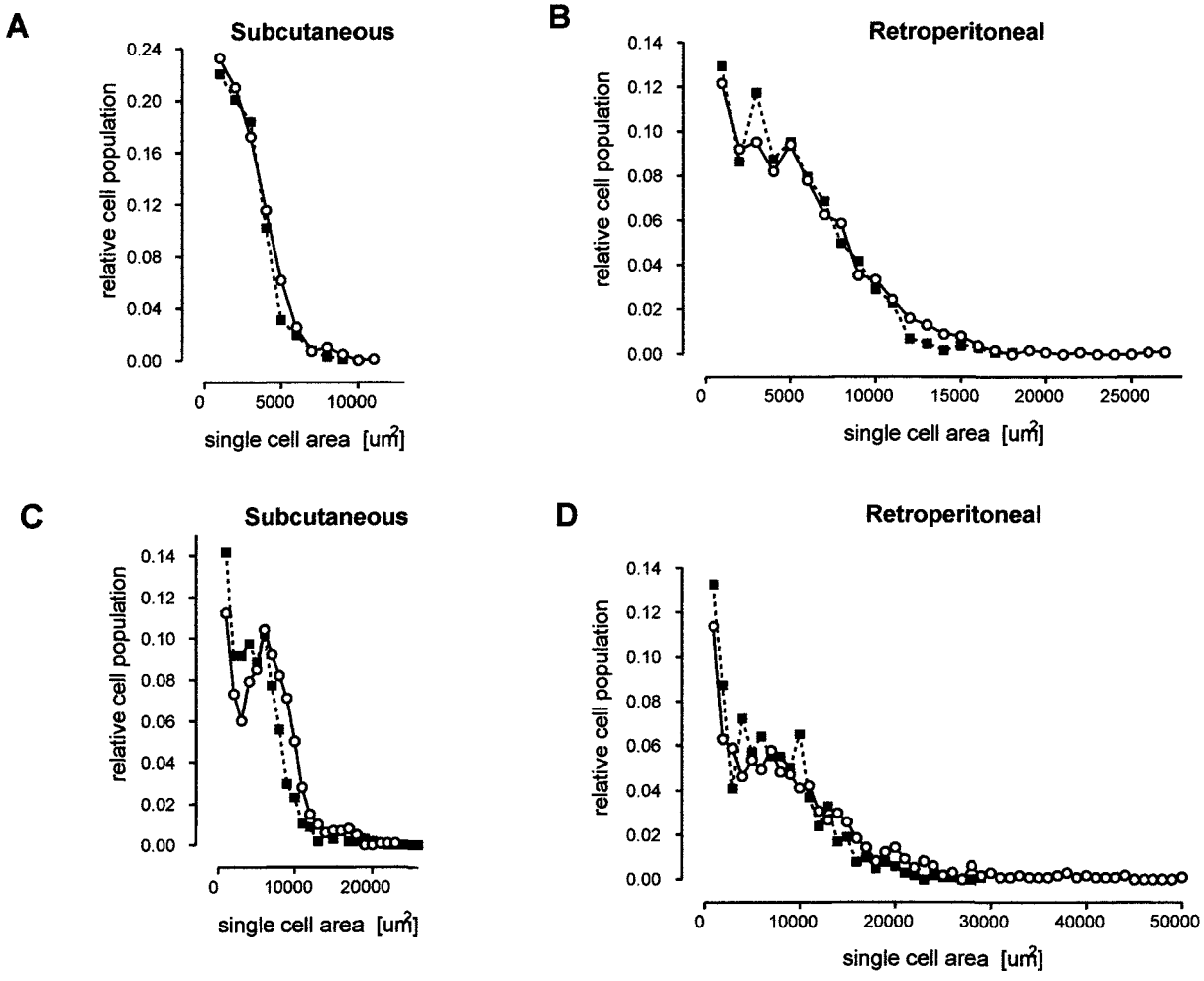

Fig. 3. Effect of CL on adipocyte size in subcutaneous and retroperitoneal adipose tissue.
Subcutaneous adipose tissue (A) and retroperitoneal adipose tissue (B) in the preventive setting; subcutaneous adipose tissue (C) and retroperitoneal adipose tissue (D) in the therapeutic setting
○ control group, ■ CL group (CL dose: 3 mg/kg).

TG contents in the liver and skeletal muscles

TG content in the liver and the skeletal muscles did not differ between the CL and control groups in either the preventive or therapeutic setting (Table 1).

WAT cellularity and weight

A comparison between the control groups at the ends of both the preventive experiment (when rats were 13 weeks old) and the therapeutic experiment (when rats were 29 weeks old) showed that in both subcutaneous and retroperitoneal fat the percentages of small adipocytes were less and the percentages of large adipocytes were more in 29-week-old rats than in 13-week-old rats (Fig. 3). In the preventive setting, the weights of subcutaneous, epididymal, mesenteric, and retroperitoneal fat were significantly lower in the CL group than in the control group (Table 2). A comparison of adipocyte populations suggested that large adipocytes (20,000 μm^2 or larger) had disappeared in the CL group, especially in the retroperitoneal adipose tissue, with an increase in the percentage of small adipocytes (Fig. 3B). Compared with the average areas of adipocytes in the control group, those in the CL group were 15% smaller ($2,297 \pm 1,751$ versus $1,958 \pm 1,571$ μm^2) in the subcutaneous fat and 11% smaller ($4,582 \pm 3,977$ versus $4,100 \pm 3,368$ μm^2) in the retroperitoneal fat. Furthermore, in the therapeutic setting the tissue weights of the mesenteric and retroperitoneal fat were significantly lower in the CL group than in the control group (Table 2). In the therapeutic setting large adipocytes (30,000 μm^2 or greater) were absent, especially in the retroperitoneal adipose tissue, whereas the percentage of small adipocytes increased (Fig.

Table 2. WAT weight and liver weight

	Preventive experiment		Therapeutic experiment	
	Control	CL316243	Control	CL316243
Subcutaneous WAT (g/100 g BW)	1.083±0.045	0.817±0.027***	1.58±0.06	1.36±0.11
Epididymal WAT (g/100 g BW)	0.797±0.037	0.605±0.018***	1.40±0.08	1.17±0.06
Mesenteric WAT (g/100 g BW)	1.007±0.040	0.677±0.027***	2.22±0.10	1.51±0.11**
Retroperitoneal WAT (g/100 g BW)	1.370±0.037	0.940±0.029***	3.07±0.12	2.36±0.21*
Liver (g/100 g BW)	3.221±0.023	3.355±0.216	3.13±0.10	3.11±0.10

*$p<0.05$, **$p<0.01$, ***$p<0.001$ versus Control

Table 3. Levels of UCP1, UCP2, and UCP3 mRNA expression in tissue

	Preventive experiment		Therapeutic experiment	
	Control	CL316,243	Control	CL316,243
BAT UCP1 (/β actin)	0.43±0.09	1.11±0.30	0.158±0.024	0.100±0.025
WAT UCP1 (/β actin)	0.028±0.018	0.056±0.016	0.0236±0.0147	0.0583±0.0259
BAT UCP2 (/β actin)	0.034±0.004	0.034±0.002	0.028±0.003	0.032±0.005
WAT UCP2 (/β actin)	0.314±0.037	0.255±0.031	0.090±0.007	0.060±0.003*
Liver UCP2 (/β actin)	0.057±0.006	0.056±0.015	0.082±0.009	0.091±0.017
Skeletal muscle UCP2 (/β actin)	0.029±0.002	0.024±0.003	0.128±0.012	0.059±0.006**
BAT UCP3 (/β actin)	0.073±0.0013	0.0130±0.0047	0.0100±0.0017	0.0052±0.0009
Skeletal muscle UCP3 (/β actin)	0.98±0.08	1.17±0.28	0.925±0.155	0.239±0.084*

*$p<0.05$, **$p<0.01$ versus Control
Relative expression levels are indicated as ratio of UCP copy number vs β actin.

3D). Compared with the average sizes of adipocytes in the control group, those in the CL group were 22% smaller in the subcutaneous fat and 26% smaller in the retroperitoneal fat.

Expression of UCP1, UCP2 and UCP3 mRNA

In the preventive setting, expression of UCP1, UCP2, and UPC3 mRNA did not differ significantly between the CL group and the control group in any tissue (Table 3). In the therapeutic setting, significant differences between the groups were seen only in expression of UCP2 in the WAT and skeletal muscles and of UCP3 in skeletal muscle, which was lower in the CL group.

Discussion

In our experiments, CL suppressed the increase in fat accumulation in 10-week-old rats (in which adipocytes grow rapidly) and reduced the accumulated fat in 26-week-old rats (in which adipocytes have already become enlarged). We therefore assumed that the therapeutic efficacy of CL in rats was not affected by the weight of accumulated fat or the size of adipocytes. A comparison of fat tissues showed a more marked reduction in intra-abdominal fat than in subcutaneous fat. However, in *in vitro* experiments, CL-induced lipolysis produced no differences between subcutaneous and intra-abdominal adipocytes (unpublished data), a result suggesting that the difference may be accounted for in part by the blood flow-enhancing properties of CL[10].

Treatment with CL decreased FFA and TG levels in both the preventive and therapeutic settings. These decreases are thought to occur when CL binds with β3-adrenoceptors in adipocytes, increasing intracellular levels of cyclic adenosine monophosphate and activating hormone-sensitive lipases to induce lipolysis[11]. The CL-induced acceleration of energy consumption via UCP resulted in immediate and local degradation of FFAs released from adipocytes. Therefore, chronic stimulation of β3-adrenoceptors induces the mobilization of fat stored as TG in white adipocytes by lipolysis and the burning (oxidation in mitochondria) of fatty acid in brown adipose tissue thermogenesis. Because FFAs released by such lipolysis and transiently increasing FFA levels in blood might be consumed in brown adipocytes and in some white adipocytes, levels of FFA and TG seems to have consequently decreased. Although insulin normally suppresses hormone-sensitive lipases in adipocytes and inhibits lipolysis[12], the antilipolytic properties of insulin may have been compromised by CL, thus further accelerating lipolysis in CL-treated rats.

Because plasma levels of leptin correlate positively with body weight and fat volume[13], we speculate that the reduction in leptin levels we observed was due to the CL-induced reduction in fat weight and to the CL-induced increase in the percentage of small adipocytes.

In the preventive setting, the increase in blood glucose levels after oral glucose loading was significantly suppressed in the CL group. The low insulin levels both at baseline and after glucose loading suggest that CL had decreased body fat volume and improved insulin resistance, thereby preventing excessive insulin secretion. However, the OGTT results in the therapeutic setting suggest that CL does not improve glucose tolerance. Furthermore, CL produced no marked changes in TG content in the liver in the preventive setting but did produce a slight but not significant increase in TG content in the therapeutic setting. In OLETF rats, increases in visceral fat associated with aging increase the amount of FFAs in the portal blood, thereby increasing intrahepatic TG levels. Thus, CL-induced lipolysis in adipose tissue might have increased the flow of FFAs into the liver. In the present study, the volume of FFAs far exceeded the liver's processing capacity in the therapeutic setting, thus causing TGs to accumulate in the liver. FFAs impair the ability of insulin to suppress hepatic glucose production[14] and might also impair hepatic glucose uptake. Injection of glucose into the portal vein of OLETF rats does not produce the decrease in FFA levels associated with endogenous insulin; instead OLETF rats exhibit increased hepatic glucose production and decreased hepatic glucose uptake compared with those in nor-

mal rats[15]. Therefore, we assume that in the therapeutic setting the CL-induced increase in FFA levels in the portal blood impairs the ability of insulin to suppress hepatic glucose production and to promote hepatic glucose uptake ; these effects might explain why glucose tolerance did not improve in the CL group, despite the maintenance of early insulin secretion after glucose loading.

An analysis of adipose tissue cellularity revealed that the size of adipocytes increased with aging, both in subcutaneous and retroperitoneal fat. In both the preventive and therapeutic settings, CL increased the percentage of small adipocytes and decreased the percentage of large adipocytes, suggesting that CL induced substantial lipolysis in large adipocytes and, as a result, reduced the size of adipocytes. Unlike WAT, in which TGs are stored, BAT processes FFAs as they are generated through the decomposition of TGs, into β-oxidation in mitochondria, and performs thermogenesis with UCP1 via tricarboxylic acid circuits and the electron transfer system[16]. In the preventive setting, CL increased rectal temperatures while increasing expression of UCP1 mRNA in BAT 2.6-fold and ectopic UCP1 in the WAT 2.0-fold over control. Therefore, we speculate that a possible mechanism of action of CL is lipolysis via β3-adrenoceptors in adipocytes and improved energy metabolism by thermogeneration via UCP1. Similarly, in the therapeutic setting, expression of ectopic UCP1 mRNA in WAT increased 2.0-fold compared with control, although expression of UCP2 and UCP3 mRNA did not increase. Although the reasons for decreases in expression of UCP2 in WAT and skeletal muscles and of UCP3 in skeletal muscles are unclear, regulation of UCP expression by FFAs[17] suggests that CL administration produces changes in lipid metabolism which then led to changes in UCP expression.

Thus, we conclude that CL suppresses the increase in fat accumulation in 10-week-old rats, whose adipocytes rapidly enlarge, and reduces accumulated fat in 26-week-old rats, whose adipocytes have already become hypertrophic. CL thus corrects abnormalities in glucose and lipid metabolism in these animals, by decomposing fat via β3-adrenoceptors in adipocytes and by improving energy metabolism through thermogeneration via UCP1.

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