

# Anti-Obesity Effects of Selective Agonists to the $\beta$ 3-Adrenergic Receptor in Dogs.

## II. Recruitment of Thermogenic Brown Adipocytes and Reduction of Adiposity after Chronic Treatment with a $\beta$ 3-Adrenergic Agonist

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**ABSTRACT.** The aim of this study was to evaluate the effectiveness of  $\beta$ 3-adrenergic agonists for the treatment and prevention of obesity in the dog. When a selective  $\beta$ 3-adrenergic agonist, CL316,243 (0.1 mg/kg), was given orally to adult beagles every day for 5–7 weeks, body weight and girth were decreased compared with control placebo-treated dogs. Gross anatomical examinations revealed no noticeable abnormalities in CL316,243-treated dogs, except an apparent decrease in abdominal fat. Immunohistochemical examination of perirenal adipose tissue showed a remarkable increase in brown adipocytes expressing a thermogenic protein, uncoupling protein (UCP). The increased expression of UCP and its mRNA in CL316,243-treated dogs was also confirmed by Western blot and reverse transcription polymerase chain reaction analyses. It was concluded that treatment with a  $\beta$ 3-adrenergic agonist stimulates UCP expression, which may lead to an increase in energy expenditure, and thereby is useful for the treatment and prevention of obesity in the dog. — **KEY WORDS:** adipose tissue,  $\beta$ 3-adrenergic receptor, canine, obesity, uncoupling protein.

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Obesity is the most common nutritional disorder, and leads to the development of various diseases such as diabetes mellitus, hypertension, atherosclerosis and osteoarthritis in humans. This is also the case in veterinary medicine for companion animals [5, 16]. The treatment of obesity is primarily dependent on either reducing energy intake via a low-calorie diet or increasing exercise, or both. In companion animals, however, it often seems difficult to achieve effective treatments, largely because owners often feel uncomfortable with them and do not follow the prescribed regimen. Recently, pharmacological treatment of obesity using specific  $\beta$ -adrenergic receptor ( $\beta$ -AR) agonists has attracted much attention in human obesity [14]. This is based on the findings that  $\beta$ 3-AR, an isoform of mammalian  $\beta$ -ARs, is expressed in adipocytes but not in other types of cells [7, 17, 19]. Thus, a specific agonist to the  $\beta$ 3-AR is expected to stimulate lipomobilization in white adipose tissue and heat production (energy expenditure) in brown adipose tissue (BAT) without serious side-effects on other organs such as the cardiovascular system, and finally to lead to reduced body fat. In fact, several  $\beta$ 3-AR agonists have been demonstrated to have potent anti-obesity effects in rodents [1, 8, 23].

In contrast to rodents, there have been few reports about the anti-obesity effects of  $\beta$ 3-AR agonists in the dog [4]. In the preceding study [22], we demonstrated the localization of the canine  $\beta$ 3-AR in adipose tissues of beagles and a potent lipomobilizing action of the  $\beta$ 3-AR agonist CL316,243. In this study, we investigated anti-obesity effects of this drug in dogs, particularly focusing on the effect on the mitochondrial uncoupling protein (UCP), which is a key molecule for heat production by BAT and thereby a

unique marker of brown adipocytes [9, 10].

### MATERIALS AND METHODS

**Animals and Treatments:** Eight adult beagle dogs of both sexes (one male and seven females) were given free access to water and fed pelleted chow at the same time (8:00–9:00) every day.

They were divided into two groups, (A and B, four dogs in each group). In the first 5-week period (weeks 0–5), group A received a capsule of CL316,243 at 0.1 mg/kg/day orally once a day, and group B received a capsule of lactose as a placebo. In the next 7-week period (weeks 6–12), the treatments were exchanged: that is, group A received a placebo and group B received CL316,243. Body weight and the girths of the abdomen and the chest were measured every 3–4 days. At the end of the experiments, dogs were deeply anesthetized with Nembutal (Dainabot, Osaka), and subjected to postmortem pathological examinations. Adipose tissues were excised from the omental, perirenal and interscapular regions. Tissue samples were fixed in 10% paraformaldehyde in PBS for histological examination and also frozen in liquid nitrogen for the detection of UCP.

Animal care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, Hokkaido University.

**Chemicals:** A highly selective  $\beta$ 3-AR agonist, CL316,243, disodium (R,R)-5 [2-(3-chlorophenyl)-2-hydroxyethyl]-aminopropyl-1,3-benzodioxole-2,2-dicarboxylate, was provided by American Cyanamid Co. (Pearl River, NY, U.S.A.) [2].

**Western blot analysis of UCP:** UCP protein was detected

by Western blot analysis as described previously [18]. Briefly, tissue samples were homogenized in 10 volumes of a solution (10 mM Tris-HCl, 1mM EDTA [pH 7.4]) for 30 sec with a Polytron. After centrifugation at 1,500 g for 5 min, the supernatant (fat-free extract) was collected and its protein concentration was determined by Lowry's method [15]. The fat-free extract (20  $\mu$ g of protein) was solubilized, subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels, and transferred to a PVDF membrane (Immobilon; Millipore, Tokyo). After blocking the membrane with 5% non-fat dried milk, it was incubated with a rabbit antiserum against rat UCP, and then with a goat anti-rabbit IgG conjugated with horseradish peroxidase (ZYMED, San Francisco, CA, U.S.A.), and finally examined with a chemiluminescence detection system (Amersham, Buckinghamshire, UK).

**Reverse transcription polymerase chain reaction (RT-PCR) of UCP mRNA:** UCP mRNA was detected by RT-PCR. The upstream (5'-GTGAAGGTCAGAATGCAAGC-3') and downstream (5'-AGGGCCCCCTTCATGAGGTC-3') primers were designed based on the published nucleotide sequence of rat UCP cDNA [3, 20]. Total RNA was prepared from the tissues using TRIzol (Gibco BRL, Tokyo). One  $\mu$ g of total RNA was reverse-transcribed at 37°C for 1 hr using 100 pmoles of oligo (dT)<sub>15</sub> as a primer in 20  $\mu$ l of 1x first-strand buffer (Gibco BRL) containing 200 U of M-MLV reverse transcriptase (Gibco BRL), 0.5 mM dNTP and 10 U of RNase inhibitor (Wako, Tokyo). PCR amplification was performed for 30 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min in 50  $\mu$ l of 1x PCR buffer (Perkin-Elmer, Branchburg, NJ, USA) containing 2.5 U Taq DNA polymerase (Perkin-Elmer), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, and 0.5  $\mu$ M of each primer. The RT-PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide.

**Immunohistochemistry:** The tissues fixed in 10% paraformaldehyde in PBS were dehydrated in ethanol, paraffin-embedded, and cut into 4  $\mu$ m-thick sections. The dewaxed sections were incubated in 0.5% periodic acid to inhibit endogenous peroxidase activity, and then with 10% normal goat serum, the rabbit antiserum against rat UCP, goat anti-rabbit IgG, and finally with avidin-biotin-peroxidase complex (Nichirei, Tokyo) according to the conventional ABC method. The sections were also counterstained with hematoxylin and examined by light microscopy.

**Blood Analysis:** Blood samples were collected before, and 5 and 12 weeks after the start of the experiment from the jugular vein into tubes containing an anticoagulant, heparin sodium (Novo Nordisk, Bagsvaerd, Denmark). After centrifugation, plasma was stored at -20°C until analysis. Analysis of blood biochemical parameters was performed with an automated analyzer (SPOTCHEM; Kyoto Daiichi Kagaku, Kyoto).

**Data analysis:** All values are given as mean  $\pm$  SE. Student's *t* test was used to compare the differences in body weight, girth and biochemical data between the groups.

## RESULTS

**Effects of CL316,243 on body weight and girth:** Eight beagles were divided into two groups (A and B), and given either CL316,243 (0.1 mg/kg) or lactose as a placebo orally once a day. The initial body weight and girth of the abdomen of group A were 13.0  $\pm$  0.9 kg and 45.9  $\pm$  1.5 cm, respectively, and those of group B were 12.1  $\pm$  0.8 kg and 45.3  $\pm$  0.5 cm. In the first phase of the experiment during weeks 0–5, group A was given CL316,243 and group B was given the placebo. As shown in Fig. 1, group B given the placebo gained weight and girth gradually, whereas the CL316,243-treated group A showed no body weight gain and a small reduction of girth. After 5 weeks, the body weight and girth of group A were lower than those of group B ( $p=0.058$  for body weight and  $p<0.05$  for girth). Food intake was also measured during the first phase. The mean energy intake of group A (381.6  $\pm$  52.7 J/day/kg) seemed to be smaller than in group B (415.1  $\pm$  33.1 J/day/kg), but the difference was not statistically significant ( $p=0.61$ ). In the next 7-week period from weeks 6 to 12 (second phase), the treatments were exchanged; group A received the placebo, and group B CL316,243. Group A gained weight and girth, whereas group B lost them. Figure 2 summarizes the changes in body weight and girth during the first and second phases of the experiment. There was a tendency for daily treatment with CL316,243 to suppress the gains of weight

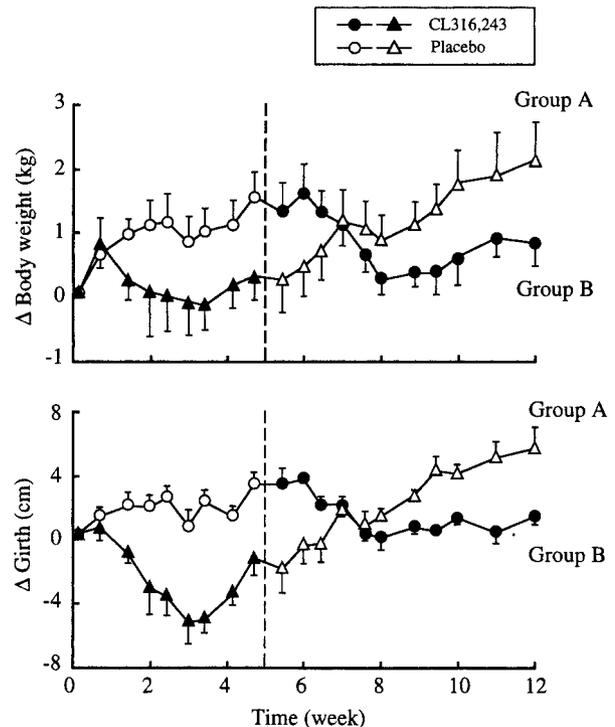


Fig. 1. Effects of daily treatment with CL316,243 (●, ▲) or a placebo (○, △) on body weight and girth in beagles. Body weight and girth are expressed as the differences from those at the start of the experiment. Values are mean  $\pm$  SE for 4 dogs.

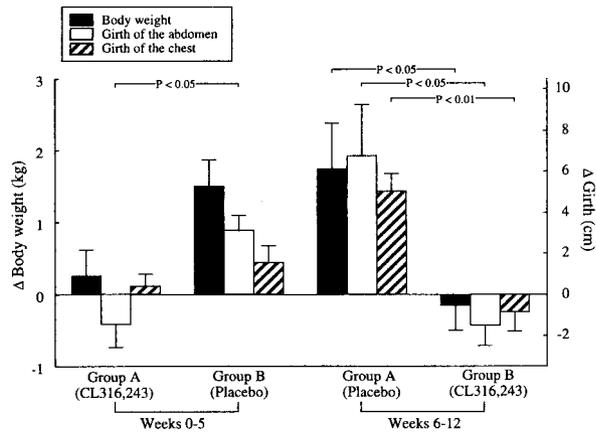


Fig. 2. Summary of the effects of CL316,243-treatment on body weight and girth gains. Values are the gains of body weight and girth during the first (weeks 0–5) and the second (weeks 6–12) phases of treatment. Values are mean  $\pm$  SE (n=4).

and girth in adult dogs.

*Effects of CL316243 on histological and biochemical features of adipose tissue:* There was no significant difference in blood biochemistry data before the experiment and at the end of the first or second phase of the experiment (Table 1). At the end of the experiment, fat pads were collected from various regions. In postmortem examinations, no apparent difference in gross appearance of individual organs, except fat pads, was found between the two groups. The fat pads of various sites of CL316,243-treated dogs (group B) were small and appeared light brown compared with those of placebo-treated dogs (group A).

Histologically, the adipose tissues of group A were composed of unilocular cells filled with a single large lipid droplet, typical of white adipocytes (Fig. 3-A). In contrast, many multilocular cells that looked like brown adipocytes were observed in the tissues of group B (Fig. 3-B). Unilocular cells were also found but their size seemed smaller than those of group A. Thus, the adipose tissue of group A contained a smaller amount of triglyceride. To determine whether the multilocular cells of the CL316,243-treated group were brown adipocytes, tissue sections were stained with an anti-UCP antibody. As shown in Fig. 3, all unilocular cells were negative for UCP, while the multilocular cells were strongly stained with the anti-UCP antibody. Thus, CL316,243-treated dogs had numerous typical brown adipocytes in the fat pads.

To confirm the presence of brown adipocytes having UCP, the expression of protein and mRNA of UCP was examined by Western blot and RT-PCR analyses. The upper panel of the Fig. 4 shows the Western blots of crude homogenates from fat pads of various sites. In placebo-treated dogs, a band of 32 kDa corresponding to rat UCP was found only in perirenal fat pads. No bands were detected in omental and interscapular fat pads. In CL316,243-treated dogs, however, a clear UCP signal was detected in all these fat pads. Essentially the same effect of CL316,243-treatment on UCP expression was found when its mRNA was analyzed by the RT-PCR method (Fig. 4): that is, a clear PCR product with the expected size (196bp) was detected in the samples from CL316,243-treated dogs.

DISCUSSION

The present results demonstrated that dogs treated with

Table 1. Effect of CL316,243-treatment on the blood biochemical parameters of beagles

	Week 0		Week 5		Week 12	
	Group A	Group B	Group A (CL316,243)	Group B (Placebo)	Group A (Placebo)	Group B (CL316,243)
AST (IU/l)	17.0 $\pm$ 2.1	14.5 $\pm$ 2.3	18.0 $\pm$ 2.1	18.8 $\pm$ 4.8	12.5 $\pm$ 1.2	13.3 $\pm$ 2.0
ALT (IU/l)	31.8 $\pm$ 9.9	32.5 $\pm$ 3.9	48.3 $\pm$ 5.2	62.5 $\pm$ 33.9	32.0 $\pm$ 8.2	32.8 $\pm$ 7.8
BUN (mg/dl)	18.8 $\pm$ 0.9	20.0 $\pm$ 3.8	18.0 $\pm$ 0.6	16.3 $\pm$ 1.4	16.3 $\pm$ 1.9	16.0 $\pm$ 1.4
Cre (mg/dl)	1.1 $\pm$ 0.7	0.1 $\pm$ 0.1	0.5 $\pm$ 0.0	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1	0.1 $\pm$ 0.0
Glc (mg/dl)	109.3 $\pm$ 2.1	108.0 $\pm$ 3.8	119.0 $\pm$ 3.4	118.0 $\pm$ 4.4	120.5 $\pm$ 4.7	119.3 $\pm$ 3.0
T-Chol (mg/dl)	225.5 $\pm$ 7.1	165.0 $\pm$ 28.0	278.0 $\pm$ 45.7	181.3 $\pm$ 38.4	240.0 $\pm$ 35.0	204.3 $\pm$ 24.6
TG (mg/dl)	34.0 $\pm$ 4.2	29.0 $\pm$ 3.3	38.8 $\pm$ 4.9	42.8 $\pm$ 6.8	45.0 $\pm$ 6.6	50.5 $\pm$ 4.1

Blood samples were taken before, and 5 and 12 weeks after the start of the experiment.

Values are mean  $\pm$  SE (n=4).

Abbreviations: AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; BUN, Blood urea nitrogen; Cre, Creatine; Glc, glucose; T-Chol, Total cholesterol; TG, Triglyceride.

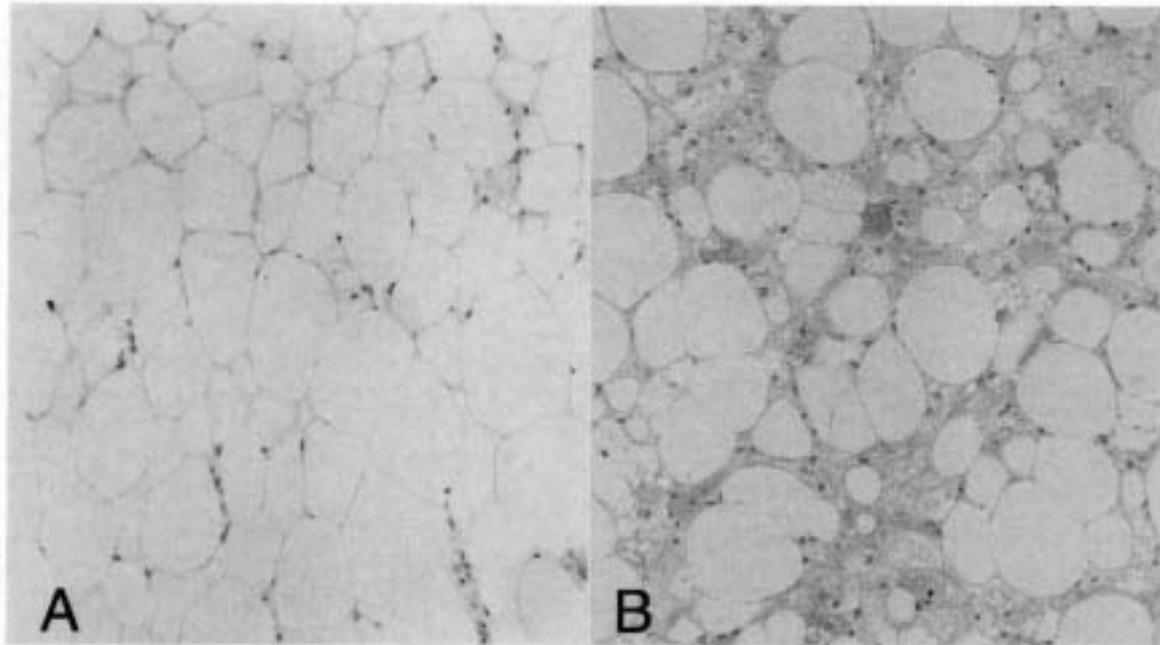


Fig. 3. Immunohistochemical detection of UCP in adipose tissue. Sections of perirenal adipose tissue of dogs treated with a placebo (A) and CL316,243 (B) were stained by a rabbit antiserum against rat UCP. Magnification,  $\times 200$ .

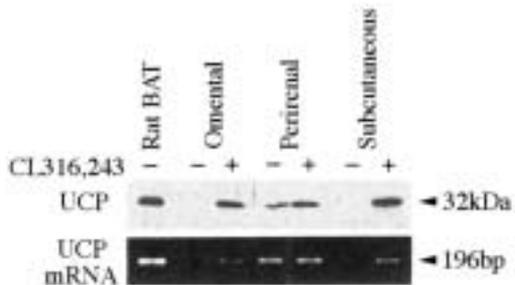


Fig. 4. Western blot and RT-PCR analyses of UCP in dog adipose tissues. Upper panel: Western blot analysis of the crude extracts of fat pads obtained from various regions of dogs treated with CL316,243 (+) and a placebo (-). Lower panel: RT-PCR analysis of total RNA extracted from fat pads of various regions of dogs treated with CL316,243 (+) and the placebo (-). Samples from rat interscapular brown fat (BAT) were used as a positive control.

CL316,243 once daily showed a tendency for reduction of body weight and girth. Champigny *et al.* [4] also reported similar effects of another  $\beta$ 3-AR agonist, ICI D7114, in dogs, although they gave the drug twice daily for a longer period and at larger dosages. In the preceding paper [22], we compared the acute lipomobilizing effects of these two drugs, and found that CL316,243 was more effective than ICI D7114 when given at the same dose. It should be noted that decreases in body weight and girth do not necessarily reflect reduced content of body fat. However, postmortem examinations confirmed that fat pads from various sites of CL316,243-treated dogs were smaller and less pale than

those of placebo-treated dogs. Therefore, it can be concluded that CL316,243 has a potent anti-obesity effect in the dog, as in rodents [8, 23].

The anti-obesity effects of  $\beta$ 3-AR agonists may be due to either reduced food intake or increased energy expenditure, or both. In fact, the mean food intake of the drug-treated dogs was reduced by about 8%, although the change was not statistically significant. In addition to such a weak effect on food intake,  $\beta$ 3-AR agonists have a potent lipomobilizing effect as reported in the preceding paper [22]. However, it should be noted that lipomobilization from accumulated body fat in white adipose tissues would not necessarily cause a loss of body fat, because the released fatty acids must be metabolized in other tissues and dissipated as heat, the final form of energy. One of the likely mechanisms for energy dissipation is metabolic heat production in BAT [21]. In fact, stimulation of BAT thermogenesis by  $\beta$ 3-AR agonists is well established in rodents [8, 23]. The stimulatory action of  $\beta$ 3-AR agonists on BAT thermogenesis is based on the acute activation of lipolysis and subsequent uncoupling of mitochondrial oxidative phosphorylation by UCP, and also on sustained tissue hyperplasia associated with increased expression of UCP [18, 23]. We [23] demonstrated that chronic treatment of obese mice with a  $\beta$ 3-AR agonist caused ectopic expression of UCP in fat pads that were usually considered to be composed of white adipose tissue. The present results clearly indicated that this was also the case in the dog: that is, UCP was detected only in perirenal fat pads in placebo-treated dogs, but chronic CL316,243-treatment induced UCP in fat pads not only in perirenal but also in retroperitoneal and subcutaneous (interscapular)

regions. Histochemical examinations revealed that these fat pads contained numerous multilocular cells positive for UCP that were indistinguishable from typical brown adipocytes. The retroperitoneal and subcutaneous fat pads of placebo-treated dogs were composed exclusively of typical unilocular white adipocytes and were always negative for UCP. Thus, CL316,243-treatment resulted in the appearance of well-differentiated brown adipocytes expressing UCP in adipose depots usually considered to be white fat, and thereby an increase in the total capacity for thermogenesis in dogs, as in rodents [8, 23]. The increased heat production would contribute to the weight loss of dogs treated with CL316,243.

In conclusion, CL316,243-treatment reduced body fat by stimulating both lipomobilization from white adipose tissue and heat production in BAT, and also probably by reducing food intake. Since the CL316,243-treatment produced no significant changes in blood biochemistry data or in the gross appearance of organs except fat pads, it is expected that  $\beta_3$ -AR agonists will be useful drugs without serious side effects for the treatment and prevention of obesity in the dog.

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