Anti-obesity and anti-diabetic effects of CL316,243, a highly specific \( \beta_3 \)-adrenoceptor agonist, in Otsuka Long-Evans Tokushima Fatty rats: induction of uncoupling protein and activation of glucose transporter 4 in white fat

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Abstract

The anti-obesity and anti-diabetic effects of a highly specific \( \beta_3 \)-adrenoceptor agonist, CL316,243 (CL; \( \beta_1; \beta_2; \beta_3 = 0:1:100,000 \)), were investigated in Otsuka Long-Evans Tokushima Fatty (fatty) and Long-Evans Tokushima Otsuka (control) rats. Daily injection of CL (0·1 mg/kg, s.c.) to these rats (10 weeks old) for 14 weeks caused a significant reduction in body weight (fatty, 27%; control, 15%), associated with a marked decrease in fat pad weight (inguinal: fatty, 60%; control, 36%; retroperitoneal: fatty, 75%; control, 77%) without affecting food intake. The levels of uncoupling protein mRNA and protein levels of uncoupling protein (UCP), as well as guanosine 5’-diphosphate-binding (a reliable index of thermogenesis) in brown adipose tissue, were lower in the fatty than in the control rats. However, after CL treatment, these parameters in brown adipose tissue increased significantly 2- to 3-fold in both groups. Furthermore, uncoupling protein was induced in white adipose tissue as well as in brown adipose tissue. The fatty rats showed hyperglycemia and hyperinsulinemia during the glucose tolerance test, but CL ameliorated these parameters. These findings suggest that decreased thermogenesis in brown adipose tissue may be one of the causes of obesity in the fatty rats and that administration of CL prevents obesity by decreasing white fat mass, by activating brown adipose tissue thermogenesis, and by inducing uncoupling protein in white adipose tissue. Furthermore, CL treatment may inhibit diabetes mellitus by ameliorating obesity and by activating glucose transporter 4 in white adipose tissue and brown adipose tissue.

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Introduction

\( \beta_1 \)-adrenoceptors, which are different from known \( \beta_1 \)- and \( \beta_2 \)-adrenoceptors, and \( \beta_3 \)-adrenoceptor mRNA are present in human white adipose tissue and brown adipose tissue (1, 2). The human \( \beta_3 \)-adrenoceptor has recently been shown to be functionally active by in vitro and in vivo studies (3, 4). Furthermore, the Trp64Arg allele of the \( \beta_3 \)-adrenoceptor is associated with abdominal obesity, insulin resistance syndrome, an earlier onset of non-insulin-dependent diabetes mellitus (NIDDM), a lower resting metabolic rate and the difficulty of body weight loss in obese subjects (5–8). However, because \( \beta_3 \)-adrenoceptor agonists (9) that stimulate lipolysis in white adipose tissue and activate brown adipose tissue could emerge as effective drugs for the treatment of obesity and diabetes in obese persons, the study and development of such substances are being actively pursued (10, 11). In this study, the anti-obesity and anti-diabetic effects of a \( \beta_3 \)-adrenoceptor agonist (CL316,243 (CL); \( \beta_1; \beta_2; \beta_3 = 0:1:100,000 \)) (12–14) were investigated in Otsuka Long-Evans Tokushima Fatty (OLETF; fatty) and Long-Evans Tokushima Otsuka (control) rats (15, 16). The characteristic features of the fatty rats are (i) late onset of hyperglycemia (after 18 weeks of age), (ii) a chronic course of disease, (iii) mild obesity, and (iv) inheritance by males. These clinical and pathological features of disease in the fatty rats resemble those of human NIDDM. The control rats were obtained by different original matings from those for the fatty rats, but both strains originated from the same colony of Long-Evans rats.

Materials and methods

Chemical

CL316,243 (CL), disodium (R,R)-5-[2-(3-chlorophenyl)-2-hydroxyethyl]-aminopropyl]-1,3-benzodioxole-2,
2-dicarboxylate (5), was provided by the American Cyanamid Co. (Pearl River, NY, USA).

**Experiment 1**

Forty male fatty and forty control rats obtained from Otsuka Pharmaceutical (Tokushima, Japan) at the age of 10 weeks were housed in metal mesh cages at 22±2 °C under a 12-h light:12-h darkness cycle, and given free access to laboratory chow and tap water. The rats were divided into those given a subcutaneous injection of CL (0.1 mg/kg) dissolved in distilled water and those given an injection of distilled water only as a control once daily. After 14 weeks (24 weeks old), weights of body, inguinal and retroperitoneal white adipose tissues and brown adipose tissue, and guanosine 5’-diphosphate (GDP)-binding in brown adipose tissue mitochondria (a reliable index of brown adipose tissue thermogenesis) (17, 18), were measured. Glucose transporter 4 (GLUT4) (19–21) mRNA, its protein and uncoupling protein (22, 23) mRNA and the amount of protein were also measured in brown adipose tissue, white adipose tissue and gastrocnemius muscle (by Northern and Western blot analyses). Immuno-histochemistry was also carried out using an anti-uncoupling protein antibody.

**GDP-binding** Interscapular brown adipose tissue samples were rapidly removed, weighed and placed in ice-cold sucrose buffer. For preparation of mitochondria, samples (4 individual samples per group) were homogenized in an ice-cold medium (pH 7.2) containing 250 mmol/l sucrose and 5 mmol/l N-Tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES). The mitochondria in the sample were isolated by differential centrifugation according to the procedure described by Cannon and Lindberg (24). The mitochondrial protein content was estimated by the method of Lowry et al. (25). Cytochrome c oxidase activity in brown adipose tissue homogenates and in isolated mitochondria were measured spectrophotometrically with a double-beam spectrophotometer (UV-140–02, Shimadzu, Kyoto, Japan) at 25 °C in 1 mmol/l of a medium consisting of 100 mmol/l KH$_2$PO$_4$, 1 mmol/l EDTA and 30 µmol/l reduced cytochrome c after treatment of the homogenates with 1% Lubrol by the method of Yonetani and Ray (26). Recovery of mitochondrial cytochrome c oxidase from brown adipose tissue homogenates was determined, and used for the calculation of total mitochondrial protein and GDP-binding per brown adipose tissue depot. The percentage of total homogenate cytochrome c oxidase recovered in mitochondrial preparations was 36.0±2.5% (distilled water-treated fatty rats), 38.9±3.7% (CL-treated fatty rats), 39.8±2.2% (distilled water-treated control rats) and 39.9±3.5% (CL-treated control rats), with no significant difference between these values. Mitochondrial GDP-binding was determined by Nicholl’s method (27).

The mitochondria were incubated at 20 °C in 0.5 ml of medium containing 48100 Bq [3H]GDP, 4551 Bq [14C]sucrose, 100 µmol/l potassium atractylloside, 20 mmol/l TES (pH 7.1), 10 mmol/l choline chloride and 5 µmol/l rotenone. After 7 min incubation, 0.4 ml of medium containing 0.26 mg mitochondrial protein was withdrawn and filtered through a nitrocellulose membrane filter with a pore size of 0.45 µm (Sartorius, Göttingen, Germany). [14C] and [3H] radioactivities of the filters were measured by scintillation spectrometry (Packard, Downers Grove, IL, USA). The volume of medium trapped on the filter was calculated from the radioactivity of [14C]sucrose.

**Northern blot analysis** Total RNA (6 individual samples per group) was extracted from 0.1-1 g of tissue using TRIzol (Gibco BRL, Tokyo, Japan) and the concentration determined from the absorbance at 260 nm. Total RNA (20 µg from brown adipose tissue, 40 µg from other tissue) was separated on a 1.5% agarose/formaldehyde gel, and transferred to and fixed on a nylon membrane. A 488 bp uncoupling protein cDNA probe corresponding to the coding region of rat uncoupling protein (28) was prepared by digesting whole uncoupling protein cDNA (a gift from Dr D Ricquier, CNRS, Meudon, France) with BamHI. The uncoupling protein probe and GLUT4 cDNA (a gift from Dr G I Bell, University Chicago, USA) were labeled with α-[32P]dCTP (ICN, Irvine, CA, USA). The blots were hybridized to the labeled probes at 42 °C for 20 h in the presence of 500 µg/ml salmon sperm DNA, and exposed to an X-ray film for autoradiography and an imaging plate of BAS1000 (Fuji Film, Tokyo, Japan) for quantitative analysis.

**Western blot analysis** Each tissue (6 individual samples per group) was homogenized in 5–10 volumes of a solution containing 10 mmol/l Tris–HCl and 1 mmol/l EDTA (pH 7.4) for 30 s with a Polytron. After centrifugation at 1500 g for 5 min, the fat cake was discarded, and the infranat (fat-free extract) was used for assay of protein (25) and cytochrome c oxidase activity (26). Uncoupling protein and GLUT4 protein in the fat-free extract was measured by Western blot analysis as described previously (29). Briefly, the fat-free extracts (10 µg protein of brown adipose tissue and muscle, 20 µg of white adipose tissue) were solubilized, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose filter. After blocking with 5% non-fat dry milk, the filter was incubated with a rabbit antiserum against rat uncoupling protein or GLUT4. The rabbit antiseras against rat uncoupling protein and GLUT4 were prepared by immunizing purified rat uncoupling protein (30) and a 12-amino acid peptide corresponding to GLUT4 (31) respectively, coupled with keyhole limpet hemocyanin. Then the filter was incubated with [125I]protein A (ICN). The dry blot was exposed to an X-ray film for
Table 1 Effects of CL316,243 on body weight, food intake, inguinal WAT weight, retroperitoneal WAT weight, mitochondrial protein content in IBAT and specific and total GDP-binding in IBAT mitochondria, in the fatty and the control rats. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th>Fatty rats</th>
<th>F-values</th>
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<tbody>
<tr>
<td></td>
<td>Distilled water-treated</td>
<td>CL316,243-</td>
<td>Between genotype</td>
</tr>
<tr>
<td></td>
<td>CL316,243-</td>
<td>treated</td>
<td>(f1, f2)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>454.8 ± 6.3</td>
<td>385.5 ± 7.9(^b)</td>
<td>16:54</td>
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<td></td>
<td>20.3 ± 0.8</td>
<td>20.4 ± 1.0</td>
<td>(1, 20)</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>7.66 ± 0.39</td>
<td>4.89 ± 0.06(^b)</td>
<td>15:00 ± 1.20(^a)</td>
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<td>4.64 ± 0.31</td>
<td>1.08 ± 0.22(^b)</td>
<td>1.63 ± 0.21(^a)</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td></td>
<td>256.0 ± 12.5(^b)</td>
<td>227.5 ± 74.8(^a)</td>
</tr>
<tr>
<td>WAT weight (g)</td>
<td>4.74 ± 0.43</td>
<td>5.96 ± 0.42</td>
<td>258.6 ± 20.9(^a)</td>
</tr>
<tr>
<td>IBAT weight (mg)</td>
<td></td>
<td>610.5 ± 67.0(^b)</td>
<td>407.4 ± 16.2(^b)</td>
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<tr>
<td>Mitochondrial</td>
<td></td>
<td></td>
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<tr>
<td>protein content</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>in IBAT (mg/IBAT)</td>
<td>444.4 ± 30.5</td>
<td>610.5 ± 67.0(^b)</td>
<td></td>
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<tr>
<td>Specific GDP-</td>
<td></td>
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<td></td>
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<tr>
<td>binding in</td>
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<tr>
<td>IBAT mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pmol/mg mitoprotein)</td>
<td>2073.1 ± 111.6</td>
<td>3681.1 ± 475.5(^b)</td>
<td>408.3 ± 82.3(^b)</td>
</tr>
<tr>
<td>Total GDP-binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in IBAT mitochondria (pmol/IBAT)</td>
<td>2073.1 ± 111.6</td>
<td>3681.1 ± 475.5(^b)</td>
<td>408.3 ± 82.3(^b)</td>
</tr>
</tbody>
</table>

WAT, white adipose tissue; IBAT, interscapular brown adipose tissue.

Recovery of mitochondrial cytochrome c oxidase from IBAT homogenates was determined and used for the calculation of total mitochondrial protein and total GDP binding per IBAT depot.

\(^a\) P < 0.05, significant difference between the fatty and control rats in the distilled water or CL316,243 group. \(^b\) P < 0.05, significant effect of drug within genotype.

**Immunohistochemistry** The fixed tissues (6 individual samples per group) were dehydrated in ethanol, paraffin-embedded and cut into 7 mm-thick sections. The dewaxed sections were incubated in 0.3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity, and then with 10% normal goat serum, using the rabbit antisera against rat uncoupling protein (diluted 500 times) (32), goat anti-rabbit IgG (diluted 400 times; Vector Laboratories, Burlingame, CA, USA), and finally with avidin-biotin-peroxidase complex (Vector Laboratories), according to the conventional ABC method. The sections were also counter-stained with hematoxylin.

**Data analysis**

Data are presented as means ± S.E.M. and were analyzed by one-way or two-way ANOVA. After justification by the ANOVA, Bonferroni t-test was performed. A P value of <0.05 was considered to indicate statistical significance.

**Results**

**Experiment 1**

Body weight and inguinal and retroperitoneal white adipose tissue weights were greater in the fatty than in the control rats. CL significantly reduced the body weight and the white adipose tissue weights of both the fatty and control rats. Food intake was about 20% greater in the fatty than in the control rats. CL had no effect on food intake. The interscapular brown adipose tissue mass was smaller in the fatty than in the control rats, while CL decreased brown adipose tissue weight in both groups. Cytochrome c oxidase activity of brown adipose tissue mitochondria did not differ significantly between the fatty (160·2 ± 15·0 μmol/min/depot) and the control (155·6 ± 13·2) rats. CL markedly (P<0.01) increased this activity in both groups (203·5 ± 21·9 vs 193·8 ± 18·8 respectively). The total brown adipose tissue mitochondrial protein content was lower in the distilled water-treated fatty than the corresponding
control rats. CL significantly increased this parameter in the fatty rats. Specific and total GDP-binding in brown adipose tissue mitochondria were lower in the distilled water-treated fatty than the corresponding control rats. Treatment with CL markedly increased both specific and total GDP-binding in brown adipose tissue mitochondria in both the fatty and the control rats (Table 1). Fig. 1 shows the contents of uncoupling protein mRNA in brown and white adipose tissues. In both fatty and control rats treated with distilled water, the uncoupling protein signal was detected in brown adipose tissue, but not in white adipose tissue. In contrast, in rats treated with CL, a clear band smaller than 18S was detected only in brown adipose tissue but also in inguinal and retroperitoneal fat pads. These findings suggest that the mRNA of uncoupling protein is present not only in brown adipose tissue but also in white adipose tissue in rats treated with CL. Fig. 2 shows the amount of uncoupling protein in brown adipose tissue and white adipose tissue. The protein (10 µg protein of brown adipose tissue, 20 µg protein of white adipose tissue) in the tissue extract from the fat pads of rats given CL gave a clear protein band at about 33 kDa, not only in brown adipose tissue, but also in inguinal and retroperitoneal fat pads, both in the fatty and control rats. In contrast, when the same amount of protein in the white adipose tissue of rats given distilled water was applied, no band was detected. Quantitative data of uncoupling protein mRNA and its protein are summarized in Figs 1 and 2. Although there was no difference between the two groups in amounts of GLUT4 mRNA and its protein in brown adipose tissue and white adipose tissue (Figs 3 and 4), the amount of GLUT4 protein in gastrocnemius muscle was lower in the fatty than in the control rats (Fig. 4). These parameters were increased by CL treatment in white adipose tissue of both the fatty and the control rats. However, the amounts of GLUT4 mRNA and its protein in muscle were not affected by CL treatment (Figs 3 and 4).

Macroscopically, brown adipose tissue pads of CL-treated rats appeared dark brown, while brown adipose tissue pads of the distilled water-treated control rats were whitish brown. Fat pads appeared less pale in the CL-treated fatty than in the control rats. Immunohistochemically, brown adipose tissues of CL-treated rats in both groups were stained more strongly by uncoupling protein antibody than those in distilled water-treated rats. The white adipose tissue in distilled water-treated rats was composed of unilocular cells filled with a single large lipid droplet, and was always negative for uncoupling protein. In contrast, many multilocular cells, which were positive for uncoupling protein, were found in the white adipose tissue of both the CL-treated fatty (Fig. 5) and control rats.

Figure 1 Northern blot analysis of uncoupling protein (UCP) mRNA of interscapular brown adipose tissue (IBAT) and white adipose tissue (WAT). The fatty and control rats were treated with CL316,243 or distilled water. Total RNA (20 µg from BAT, 40 µg from WAT) was used for the analysis. UCP mRNA contents were expressed relative to control IBAT (distilled water) after correcting for applied doses. Values are means±SEM for 6 rats. *P<0.05 compared with distilled water-treated group in the fatty or the control rats. ND, non-detectable.
Figure 2 Western blot analysis of uncoupling protein (UCP) protein of interscapular brown adipose tissue (IBAT) and white adipose tissue (WAT). The fatty and control rats were treated with CL316,243 or distilled water. Fat-free extracts containing 10 µg protein of BAT or 20 µg protein of WAT were used for the analysis. UCP protein contents were expressed relative to control IBAT (distilled water) after correcting for applied doses. Values are means±s.E.M. for 6 rats. *P<0.05 compared with distilled water-treated group in the fatty or the control rats. ND, non-detectable.

Experiment 2
As shown in Fig. 6, the levels of blood glucose and serum insulin were significantly higher in the fatty rats treated with distilled water for 14 weeks than those in the control rats treated with distilled water. Treatment with CL for 14 weeks decreased the blood glucose levels significantly in the fatty, but only slightly in the control rats. In addition, CL greatly reduced the serum insulin levels in the fatty rats.

Discussion
Dysfunction of brown adipose tissue has been shown to be one of the causes of obesity in various obesity models (34–36). In this study, the fatty rats also displayed characteristics of dysfunction of brown adipose tissue such as reduced GDP-binding in brown adipose tissue mitochondria and decreased uncoupling protein mRNA and uncoupling protein protein contents, suggesting that dysfunction of brown adipose

Figure 3 Northern blot analysis of GLUT4 mRNA of interscapular brown adipose tissue (IBAT), white adipose tissue (WAT) and gastrocnemius muscle. The fatty and control rats were treated with CL316,243 or distilled water. Total RNA (20 µg from BAT, 40 µg from WAT and muscle) was used for the analysis. GLUT4 mRNA contents were expressed relative to control (distilled water). Values are means±s.E.M. for 6 rats. *P<0.05 compared with distilled water-treated group in the fatty or the control rats.
Western blot analysis of GLUT4 protein of interscapular brown adipose tissue (IBAT), white adipose tissue (WAT) and gastrocnemius muscle. The fatty and control rats were treated with CL316,243 or distilled water. Fat-free extracts containing 10 µg protein of BAT or 20 µg protein of WAT and muscle were used for the analysis. Amount of GLUT4 protein was expressed relative to controls (distilled water). Values are means±SEM for 6 rats. *P<0.05, compared with distilled water-treated group in the fatty or control rats. \#P<0.05, significant difference between the fatty and the control rats in the CL316,243- or distilled water-treated group.

The present findings that CL induced body weight loss and fat pad weight loss without affecting food intake and, furthermore, ameliorated hyperglycemia and hyperinsulinemia, were consistent with our own (13, 32, 33) and others (12, 14) previous findings.

This study showed that CL, a β3-adrenoceptor agonist, induced uncoupling protein (generally considered present only in brown adipose tissue (22, 23, 37)) even in white adipose tissue. This is consistent with our previous study using yellow KK mice (32), the report of Himms-Hagen et al. (14) showing that the mesenteric and intra-abdominal white fat depots contained numerous multilocular brown adipocyte-like cells in Sprague–Dawley rats treated with CL compound, and the reappearance of uncoupling protein in adipose depots in adult dogs treated with another β3-agonist, ICI-D7114 (38). It is unlikely that the

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**Figure 4** Western blot analysis of GLUT4 protein of interscapular brown adipose tissue (IBAT), white adipose tissue (WAT) and gastrocnemius muscle.

**Figure 5** Immunohistochemical detection of uncoupling protein (UCP) in white adipose tissue of fatty rats treated with CL316,242 (left) or distilled water (right). Sections of inguinal fat pad were stained by rabbit antiserum against rat UCP. Arrows show multilocular cells which were positive for UCP. (Magnification ×80.)
signals found in these tissues are derived from other mRNA or protein, because their molecular sizes were identical to those found in brown adipose tissue. Ricquier et al. (39) reported the appearance of a false signal of uncoupling protein mRNA in rat liver by hybridization with the probes of whole uncoupling protein cDNA and its 3' non-coding region. To avoid this, we used a cDNA probe corresponding to the coding region of uncoupling protein cDNA. Immunocytochemical examinations showed that these fat pads, conventionally considered to be white fat, contained cells indistinguishable from typical brown adipocytes. The origin of brown adipocytes in the white fat could be explained principally by either recruitment of preadipocytes to the tissue or a conversion of adipocyte phenotypes from white to brown. Actually, the conversion of brown adipose tissue to white adipose tissue has been observed in various types of obese animals (13, 34, 40, 41). There is no evidence at present that uncoupling protein expressed in white adipose tissue is functionally active. In our preliminary study the GDP-binding in the inguinal white adipose tissue mitochondria of the CL-treated fatty rats was not detectable. Therefore, further examination with a more sensitive technique for measurement of thermogenesis or studies following long-term administration of CL compound to the fatty rats are needed. However, the ectopic expression of uncoupling protein may contribute to the increased energy expenditure and the effectiveness of $\beta_3$-adrenergic agonist in reducing the body fat of obese animals.

We previously reported that $\beta_3$-adrenoceptor agonists promoted insulin secretion acutely (42–44) and increased the number of insulin receptors associated with decreased fat chronically (33). GLUT4 expression and glucose utilization in brown adipose tissue are increased by adrenergic stimulation due to cold exposure (29, 45) or by agonists including $\beta_3$-agonists (21). In this study, we found that $\beta_3$-adrenoceptor agonists increased GLUT4 levels in brown and white adipose tissues, although there was no increase in the GLUT4 level in muscle. Glucose uptake in skeletal muscle can be enhanced independently of the action of insulin (46), probably through the mediation of $\beta_3$-adrenoceptors present in the tissue. Therefore, the improvement in hyperglycemia and hyperinsulinemia observed in this obese model may be related to the increased number of insulin receptors associated with the decrease in fat pads, the increase in GLUT4 content in white and brown adipose tissues and, probably, the increase in glucose uptake in muscle.

Although it remains to be examined whether the present findings can be applied to humans, they cast a new light on the treatment of obesity using $\beta_3$-adrenoceptor agonists.

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