Anti-obesity effect of CL 316,243, a highly specific β3-adrenoceptor agonist, in mice with monosodium-L-glutamate-induced obesity

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The effects of CL 316,243, a highly specific β3-adrenoceptor agonist (relative selectivities of 0.1 and 100 000 for β1-, β2- and β3-receptors, respectively), were evaluated in mice with monosodium l-glutamate (MSG)-induced obesity as well as in control mice injected with physiological saline instead of MSG. Both MSG- and saline-treated mice were divided into three groups and at 8 weeks of age received either CL 316,243 (0.1 or 1.0 mg/kg) or distilled water through a gastric tube for 2 weeks. CL 316,243 not only reduced white adipose tissue mass but also activated brown adipose tissue and systemic metabolism, and hence reduced body mass without affecting food intake. Furthermore, CL 316,243 decreased hyperglycaemia and hypertriglyceridaemia in MSG-treated mice. However, at the higher dose, CL 316,243 also increased liver triglyceride in MSG-treated mice. These observations suggest that CL 316,243 exerts an anti-obesity effect in mice with MSG-induced obesity and consequently may prove efficacious in the treatment of human obesity.

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Messenger RNA encoding the β3-adrenergic receptor is present in human white and brown adipose tissue (1). Because β3-adrenoceptor agonists (2) that stimulate lipolysis in white adipose tissue (WAT) and activate brown adipose tissue (BAT) 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 (3–5). The β3-agonists that have been developed to date have residual side effects at β1- and β2-adrenergic receptors in humans (6). We now described the effects of the β3-adrenoceptor agonist CL 316,243 (7), which is superior to conventional drugs in terms of β3-receptor specificity (relative selectivities of 0.1 and 100 000 for β1-, β2- and β3-receptors, respectively), on obesity induced by monosodium L-glutamate (MSG) in mice (8–10).

Methods

CL 316,243, or disodium (R,R)-5-[2-[(2-3-(3-chlorophenyl)-2-hydroxyethyl)-amino]propyl]-1,3-benzo[dioxole-2,2-dicarboxylate (Fig. 1) (7), was developed by Lederle Laboratories, American Cyanamid Company (Pearl River, NY).

Obesity was induced in 30 male ICR mice (Charles River Japan, Tokyo, Japan) by daily subcutaneous injection with MSG (2 g/kg body mass) (Wako Pure Chemical Industries, Tokyo, Japan) immediately after birth for five consecutive days (8–10). Physiological saline alone was injected into 30 control male ICR mice. Animals were weaned at 3 weeks of age, housed under conditions of controlled temperature (22 ± 2°C) and artificial light from 06.00 h to 18.00 h and provided with commercial powdered chow (Charles River Japan) and tap water ad libitum. At 7 weeks, both MSG- and saline-treated mice were divided into three groups. After 1 week, the first and second groups received CL 316,243 via a gastric tube at a daily dose of 0.1 mg/kg or 1.0 mg/kg, respectively, for 2 weeks. The third group received distilled water in the same manner. Daily food intake and body mass were measured. One week after the onset of treatment with CL 316,243 (or distilled water), the resting metabolic rate (RMR) was measured. After a stable baseline was achieved, although the animals were active at the time of the measurement (11), the RMR was measured for 1 h at an ambient temperature of 22°C with the use of a closed-circuit metabolic system (ACM-1; Environics, Newton, MA) (12). After treatment with CL 316,243 for 2 weeks, the animals were killed by decapitation and blood was collected for determination of blood glucose as well as serum total cholesterol and triglyceride. Blood glucose was measured by the glucose oxidase method (Fuji Medical System, Tokyo, Japan) and total cholesterol and triglyceride were measured by enzymatic methods. Interscapular BAT (IBAT) samples were rapidly removed, weighed and placed in ice-cold sucrose buffer. For preparation of mitochondria, IBAT from
two mice in each group was pooled and homogenized in an ice-cold solution containing 250 mmol/l sucrose and 5 mmol/l Tes buffer (pH 7.2). Mitochondria were isolated by differential centrifugation according to the procedure described by Cannon and Lindberg (13). The mitochondrial protein content was determined by the method of Lowry et al. (14). Cytochrome c oxidase activity in IBAT homogenates and in isolated mitochondria were measured spectrophotometrically with a double-beam spectrophotometer (UV-140-02; Shimadzu, Kyoto, Japan) at 25°C in 1 ml of a solution consisting of 100 mmol/l KH$_2$PO$_4$, 1 mmol/l EDTA and 30 µmol/l reduced cytochrome c, after treatment with 1% Lubrol as described by Yoneshani and Ray (15). Recovery of mitochondrial cytochrome c oxidase from IBAT homogenates was determined and used to calculate total mitochondrial protein and guanosine 5'-diphosphate (GDP) binding per IBAT depot. Mitochondrial GDP binding was determined by the method of Nicholls (16). The mitochondria were incubated for 7 min at 20°C in 0.5 ml of solution containing 1.30 µCi of [3H]-GDP, 0.123 µCi of [14C]sucrose, 100 µmol/l potassium atractyloside, 20 mmol/l Tes buffer (pH 7.1), 10 mmol/l choline chloride and 5 µmol/l rotenone. A 0.4-ml portion of reaction mixture, containing 0.26 µg of mitochondrial protein, was then filtered through a nitrocellulose membrane filter with a pore size of 0.45 µm (Sartorius, Göttingen, Germany). Radioactivity (14C and 3H) associated with the filters was assayed by liquid scintillation spectroscopy (Packard, Downers Grove, IL). [14C]Sucrose was included to calculate the volume of medium trapped on the filter. The masses of the liver and retroperitoneal, mesenteric and subcutaneous WAT were determined. Fat was extracted from the liver by the method of Folch et al. (17) and total cholesterol and triglyceride were assayed by enzymatic methods.

Data are presented as means ± SEM and were analyzed by one-way or two-way analysis of variance (ANOVA) and the Bonferroni t-test. A p value of <0.05 was considered to be statistically significant.

### Results

Body mass and retroperitoneal, mesenteric and subcutaneous WAT mass in the MSG groups were greater than in the saline control groups (Table 1). CL 316,243 markedly decreased body and WAT mass in a dose-dependent manner in both MSG and saline groups. The RMR was markedly increased by treatment with CL 316,243 at both doses in both MSG and saline groups (Table 2). The IBAT mass was greater in the MSG groups than in controls but was unaffected by CL 316,243 (Table 3). Cytochrome c oxidase activity of IBAT mitochondria did not differ significantly between MSG and control groups, whereas CL 316,243 markedly increased the activity in both groups. Total mitochondrial protein content and total GDP binding per IBAT depot were calculated from the recovery of cytochrome c oxidase. The total IBAT mitochondrial protein content was lower in the MSG group treated with distilled water than in the corresponding control group; CL 316,243 significantly increased this parameter in both MSG and saline groups. Although specific GDP binding in IBAT mitochondria was similar between corresponding MSG and saline control groups, total

### Table 1. Effects of CL 316,243 administration for 2 weeks on body mass and subcutaneous, mesenteric and retroperitoneal white adipose tissue (WAT) mass in monosodium-$\alpha$-glutamate (MSG)-treated obese and saline-treated control mice.

<table>
<thead>
<tr>
<th></th>
<th>Saline control mice</th>
<th>MSG obese mice</th>
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<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>CL 316,243 (0.1 mg/kg)</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>30.8 ± 0.4</td>
<td>27.5 ± 0.3⁵</td>
</tr>
<tr>
<td>Subcutaneous WAT (g)</td>
<td>0.54 ± 0.02</td>
<td>0.26 ± 0.04⁵</td>
</tr>
<tr>
<td>WAT mass (g)</td>
<td>0.33 ± 0.02</td>
<td>0.23 ± 0.02⁵</td>
</tr>
<tr>
<td>Mesenteric WAT mass (g)</td>
<td>0.77 ± 0.08</td>
<td>0.21 ± 0.06⁶</td>
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<tr>
<td>Retroperitoneal WAT (g)</td>
<td></td>
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</tbody>
</table>

* All data are presented as means ± SEM.
* b < 0.05 vs corresponding saline group.
* c < 0.05 vs distilled water.
* d < 0.05 vs CL 316,243 (0.1 mg/kg).
Table 2. Effects of CL 316,243 administration for 1 week on resting metabolic rate (RMR) in monosodium-L-glutamate (MSG)-treated obese mice and saline-treated control mice.

<table>
<thead>
<tr>
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<th>Saline control mice</th>
<th>MSG obese mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>CL 316,243 (0.1 mg/kg)</td>
</tr>
<tr>
<td>RMR (ml·min⁻¹·body wt⁻⁰·⁷⁵)</td>
<td>16.5 ± 2.2</td>
<td>23.8 ± 2.8b</td>
</tr>
</tbody>
</table>

* All data are presented as means ± SEM.

p < 0.05 vs distilled water.

GDP binding was lower in the MSG group treated with distilled water than in the corresponding control group. Treatment with CL 316,243 markedly increased both specific and total GDP binding in IBAT mitochondria at both doses in both MSG and control groups. Food consumption (g/day) was significantly (p < 0.05) lower in the MSG group treated with distilled water (3.9 ± 0.2) than in the corresponding control group (4.8 ± 0.3); CL 316,243 treatment had no significant effect on food intake (MSG groups: 4.1 ± 0.3 at 0.1 mg/kg and 4.0 ± 0.5 at 1.0 mg/kg; saline control groups: 5.1 ± 0.4 at 0.1 mg/kg and 5.2 ± 0.6 at 1.0 mg/kg).

Blood glucose concentration was higher in the MSG groups than in the control groups and CL 316,243 significantly decreased this parameter only in MSG-treated mice (Fig. 2). Total serum cholesterol was increased significantly in the MSG groups relative to control mice, but was unaffected by CL 316,243. Serum triglyceride was also increased significantly in MSG groups. However, CL 316,243 treatment reduced the serum triglyceride concentration in both MSG- and saline-treated animals. Liver mass was significantly higher in MSG groups than in control groups (Fig. 3). CL 316,243 at a dose of 1.0 mg/kg increased liver mass in MSG-treated mice. Total cholesterol in liver did not differ between MSG and control groups and was unaffected by CL 316,243. Liver triglyceride was increased in the MSG groups relative to control groups; administration of CL 316,243 at 1.0 mg/kg further increased this parameter in MSG-treated mice.

Discussion

Our results indicate that CL 316,243, a highly specific β3-adrenoceptor agonist, not only decreased WAT mass but also stimulated BAT and systemic metabolism.TableRow 3. Effects of CL 316,243 administration for 2 weeks on interscapular brown adipose tissue (IBAT) mass as well as cytochrome c oxidase activity, total protein content and specific and total guanosine 5′-diphosphate (GDP) binding in IBAT mitochondria of monosodium-L-glutamate (MSG)-treated obese and saline-treated control mice.

<table>
<thead>
<tr>
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<th>Saline control mice</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>CL 316,243 (0.1 mg/kg)</td>
</tr>
<tr>
<td>IBAT mass (mg)</td>
<td>72.8 ± 10.9</td>
<td>87.5 ± 11.2</td>
</tr>
<tr>
<td>Cytochrome c oxidase activity (µmol·min⁻¹·depot⁻¹)</td>
<td>16.2 ± 1.0</td>
<td>36.5 ± 4.3c</td>
</tr>
<tr>
<td>Mitochondrial protein content (mg/depot)</td>
<td>2.68 ± 0.06</td>
<td>3.11 ± 0.15c</td>
</tr>
<tr>
<td>Specific GDP binding (nmol/kg protein)</td>
<td>112.5 ± 11.4</td>
<td>239.2 ± 8.3c</td>
</tr>
<tr>
<td>Total GDP binding (pmol/depot)</td>
<td>301.5 ± 12.2</td>
<td>743.9 ± 26.8c</td>
</tr>
</tbody>
</table>

* All data are presented as means ± SEM.

Mitochondrial data were obtained from a pooled preparation of IBAT depots from two mice. 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. Percentages of total homogenate cytochrome c oxidase recovered in mitochondrial preparations were 30.5 ± 2.5, 37.5 ± 3.3, 39.2 ± 3.1, 30.8 ± 2.1, 36.3 ± 2.5 and 39.8 ± 3.5% in MSG + distilled water, MSG 0.1 mg/kg CL 316,243, MSG 1.0 mg/kg CL 316,243, saline + distilled water, saline 0.1 mg/kg CL 316,243 and saline 1.0 mg/kg CL 316,243, respectively.

b p < 0.05 vs corresponding saline group.

c p < 0.05 vs distilled water.

d p < 0.05 vs CL 316,243 (0.1 mg/kg).
play an important role in the increase in oxygen consumption induced by CL 316.243.

CL 316.243 treatment decreased both hyperglycemia and hypertriglyceridemia in MSG obese mice. These results are also consistent with those of previous studies with BRL 26830A in db/db mice (3) and yellow KK mice (19). Acute administration of BRL 26830A increases insulin secretion from the pancreas (18, 21, 22), whereas chronic administration of the drug enhances tissue sensitivity to insulin by, for example, increasing the number of insulin receptors (19, 23, 24). The CL 316.243-induced improvement in hyperglycemia in the present study therefore may be attributable to an increased tissue sensitivity to insulin that results from a decrease in body mass.

The CL 316.243-induced decrease in WAT mass was dose dependent and suggests that the increased lipid mobilization may contribute to liver steatosis by thereby reducing total body mass. These data are consistent with the results of previous studies of the effects of conventional $\beta_2$-agonists in ob/ob mice (2–5), db/db mice (2–5) and Zucker fa/fa rats (18). The effects of CL 316.243 were apparent at one-tenth to one-twentieth of the dose of the $\beta_2$-agonist BRL 26830A shown to be effective in our previous studies (11, 19).

With regard to the increase in the RMR induced by CL 316.243, it is unclear whether this compound has any effect on protein metabolism. Studies in rats with other $\beta_2$-agonists have shown that BRL 26830A has no effect on muscle mass, whereas BRL 35135 increases soleus muscle mass but has no effect on the mass of gastrocnemius or plantaris muscles (3). We have also observed previously (20) that BRL 35135 decreases both mass without affecting lean body mass in obese rats fed a high-carbohydrate diet. Therefore, fat may

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**Fig. 2.** Effect of CL 316.243 administration for 2 weeks on blood glucose concentration, serum total cholesterol concentration and serum triglyceride concentration in monosodium-L-glutamate (MSG)-treated obese mice and saline-treated control mice. All data are presented as means ± SEM: (•) animals treated with distilled water; (■) animals treated with CL 316.243 (0.1 mg/kg); (□) animals treated with CL 316.243 (1.0 mg/kg). *p < 0.05; †p < 0.01 vs saline: ‡p < 0.05; ‡p < 0.01 vs distilled water: †p < 0.05 vs CL 316.243 (0.1 mg/kg).

**Fig. 3.** Effect of CL 316.243 administration for 2 weeks on liver mass and the content of total cholesterol concentration and triglyceride in the liver of monosodium-L-glutamate (MSG)-treated obese mice and saline-treated control mice. All data are presented as means ± SEM: (•) animals treated with distilled water; (■) animals treated with CL 316.243 (0.1 mg/kg); (□) animals treated with CL 316.243 (1.0 mg/kg). *p < 0.05; †p < 0.01 vs saline: ‡p < 0.05 vs distilled water: †p < 0.01 vs CL 316.243 (0.1 mg/kg).

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mobilized fatty acids. Furthermore, activation of BAT metabolism, which appeared almost maximal at the lower dose (0.1 mg/kg) of CL 316,243, may explain the increase in the hepatic content of triglyceride induced by the higher dose (1.0 mg/kg) of CL 316,243 in MSG obese mice. However, CL 316,243 did not induce fatty steatosis in saline-treated control mice in the present study or in yellow KK obese and diabetic mice (Yoshida et al., unpubl. obs.).

Clinical studies on various β3-receptor agonists (25–28) have revealed that significantly larger doses are required to elicit anti-obesity or anti-diabetic effects in humans than in mice or rats. This discrepancy may be attributable to differences in the efficiency of β3-agonist-induced signal transduction between human and rodent fat cells (29). Recent evidence suggests that β3-receptors in humans are more plentiful in perirenal than omental fat, and are even less abundant in subcutaneous fat (1). Results of studies with human subcutaneous fat cells should thus be interpreted in light of the fact that these cells contain few β3-receptors (1). In addition, recent studies have revealed that mouse (30) and rat (31) β3-receptors consist of 388 and 400 amino acids, respectively, whereas the human β3-receptor comprises 402 amino acids (32).

Messenger RNA encoding the mitochondrial uncoupling protein, which was assumed to be restricted to brown fat cells, has recently been demonstrated in areas consisting of white fat cells (1). Furthermore, administration of a β3-agonist to adult dogs with small amounts of BAT restored mRNA and expression of the BAT mitochondrial uncoupling protein (33).

Our results, together with the demonstration that CL 316,243 possesses a greater β3-receptor specificity than other β3-agonists (7), indicate that studies are required to clarify the effects of CL 316,243 on human adipocytes in vitro.

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