Endocrine Journal 1997, **44** (1), 181–185

NOTE

Effect of CL316,243, a Highly Specific β_3-Adrenoceptor Agonist, on Lipolysis of Epididymal, Mesenteric and Subcutaneous Adipocytes in Rats

TSUNEKAZU UMEKAWA, TOSHIHIDE YOSHIDA, NAOKI SAKANE, AND MOTOHARU KONDO

First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto 602, Japan

Abstract. To clarify whether a β_3-adrenoceptor agonist is more lipolytic in the visceral adipocytes than in the subcutaneous adipocytes, the lipolysis induced by CL316,243, a highly specific β_3-adrenoceptor agonist (relative selectivities of 0, 1 and 100,000 for β_1-, β_2- and β_3-receptors, respectively) was investigated in adipose tissue from rats. White adipocytes were prepared from the subcutaneous, mesenteric, and epididymal white adipose tissues of male Wistar rats (weighing about 150 g). Our findings showed that lipolysis of white adipocytes was stimulated both by the non-specific β-adrenoceptor agonist, isoproterenol, and by the β_3-specific adrenoceptor agonist, CL316,243, but the lipolytic sensitivity to CL316,243 was about 10 times greater than that to isoproterenol in these three adipose tissues. Both isoproterenol and CL316,243 induced more noticeable lipolysis in the epididymal and mesenteric than in the subcutaneous adipose cells in terms of the pD_2 value [$-\log$ mol l^{-1} for EC_{50} (the concentration of an agonist giving half of its own maximum stimulation)]. These findings show that CL316,243 is more lipolytic in the visceral adipose cells than in the subcutaneous adipose cells, although epididymal adipose cells showed a high lipolytic response close to those observed in visceral adipose cells. CL316,243 may therefore be especially useful for the treatment of visceral fat type obesity related to various diseases.

Key words: Anti-obesity drug, Visceral fat, White adipose tissue, β-Adrenoceptor, Lipolytic action
(*Endocrine Journal* **44**: 181–185, 1997)

β_3-ADRENOCEPTOR agonists reportedly behave as anti-obesity drugs [1–5], because they promoted the lipolysis of white adipocytes and activated brown adipose tissue thermogenesis, consequently leading to the loss of body weight. Although reported [6, 7] in the adipocytes of small mammals, such as rats and mice, the β_3-agonist, BRL37344, is more lipolytic than the non-specific β-adrenoceptor agonist, isoproterenol, but in the adipocytes of larger mammals, such as rabbits, isoproterenol is rather more lipolytic than BRL37344 and, furthermore, in humans, the lipolytic action of BRL37344 and CGP12177 (at present the only known selective β_3-agonist in humans) was very weak or absent. On the other hand, the accumulation of visceral adipose tissue has been shown to be closely associated with diabetes mellitus, hyperlipidemia, hypertension, and arteriosclerosis [8–11]. Visceral fat has been reported to have a high metabolic activity, as compared with subcutaneous fat [11, 12]. In visceral fat type obesity, a decrease in body weight specifically reduced visceral fat and improved glucose intolerance and hyperlipidemia [13]. The m-RNA expression of β_3-adrenoceptors in human adipose tissue also was higher in visceral than in subcutaneous adipose tissue [14]. These findings

Received: April 19, 1996
Accepted: August 19, 1996
Correspondence to: Dr. Toshihide YOSHIDA, First Department of Internal Medicine, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-Kawaramachi, Kamikyo-ku, Kyoto 602, Japan

lead to the hypothesis that β_3-adrenoceptor agonists are more lipolytic in visceral adipose cells than in subcutaneous adipose cells. In this study, we therefore investigated this hypothesis by using CL316,243 (β_1: β_2: β_3 = 0:1:100,000) [2], a specific β_3-adrenoceptor agonist in rats.

Materials and Methods

CL316,243, disodium (R,R)-5-[2-[[2-3(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate, was developed by Lederle Laboratories, American Cyanamid Company (Pearl River, N.Y., USA) [2]. Isolated adipocytes in 60 male Wistar rats (6 weeks old, about 150 g) were prepared from the fat specimens by collagenase treatment with slight modifications [15, 16] of Rodbell's original method [17]. Subcutaneous, mesenteric, and epididymal adipose tissues (about 5 g, respectively) were removed from the rats. Collagenase [Type II (Lot83H6844: Sigma Chemical Co., St.Louis, MO, USA)] was dissolved in modified Krebs-bicarbonate (m-KRB) solution (mM: NaCl 118.4; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 1.25; $NaHCO_3$, 25.0; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 20.0; KH_2PO_4, 1.2; bovine serum albumin, 2%w/v; pH, 7.4) aerated with a gas mixture (5% CO_2, 95% O_2) for 30 min and the concentration of collagenase was adjusted to 30 mg/15 m*l*. Fat tissue was suspended in m-KRB solution (15 m*l* per 5 g), mixed with the same volume of collagenase solution, and after replacing of air with the gas mixture, was covered and incubated at 37 °C with mild shaking for 50 min. The cell suspension was filtered through gauze and centrifuged (400 g × 1 min) at room temperature. The fluid in the lower layer was aspirated. By a similar procedure, adipocytes were washed twice, and the cell count was adjusted with a hemacytometer to a final concentration of 5×10^5 cells/m*l*. The cell suspension was placed in polyethylene tubes (450 μl per tube), preincubated for 5 min with 25 μl of m-KRB solution, and mixed with 25 μl of isoproterenol (Isop) or CL316,243 (CL) (concentration at the time of measurement of glycerol: Isop or CL, 10^{-4}-10^{-11}M/*l*) to a total volume of 500 μl. After replacing the air with a gas mixture (5% CO_2, 95% O_2), the cell suspension was covered and incubated at 37°C for 90 min. After ice-cooling for 10 min, 200 μl of 1N NaOH was added for neutralization. After centrifugation (1500 × g, 4°C, 10 min), the middle layer was obtained and used as samples. Glycerol was measured using a F-kit Glycerol [18] (Boehringer Mannheim, Mannheim, Germany). Dose-response curves for glycerol release were used to determine the concentrations of agonists giving half of their own maximum stimulation (EC_{50}). The value for this concentration represents pD_2 ($-\log$ mol l^{-1} for EC_{50}). Intrinsic activities of the lipolytic agents were determined as the percentage of the maximal Isop response for each experiment separately.

Chemicals

Dl-isoproterenol hydrochloride (Isop) was purchased from Wako Pure Chemicals (Osaka, Japan), and albumin bovine essentially globulin free lyophilized powder (Lot 79F 9302) and Hepes from Sigma Chemical Co. (St. Louis, MO, USA). Various reagents used for a phosphate solution were purchased from Wako Pure Chemicals. All chemicals were dissolved in and adjusted with saline.

Statistics

Data were presented as the means ± SEM and analyzed by one-way or two-way analysis of variance (ANOVA). After justification by ANOVA, the Bonferroni *t*-test was performed.

Results

As shown in Fig.1(A), isoproterenol (Isop) induced lipolysis at a concentration of more than 10^{-9} M and showed 100% lipolysis at more than 10^{-7} M in subcutaneous, mesenteric and epididymal adipocytes. The pD_2 value for Isop was 7.72 ± 0.06 in subcutaneous, 8.02 ± 0.07 in epididymal and 7.96 ± 0.05 in mesenteric adipocytes. The lipolytic action of Isop was significantly ($P<0.01$) greater in the epididymal and mesenteric than in the subcutaneous adipocytes in terms of the pD_2 value, but no significant difference was observed between the epididymal and mesenteric adipocytes.

As shown in Fig. 1 (B), CL316,243 (CL) induced

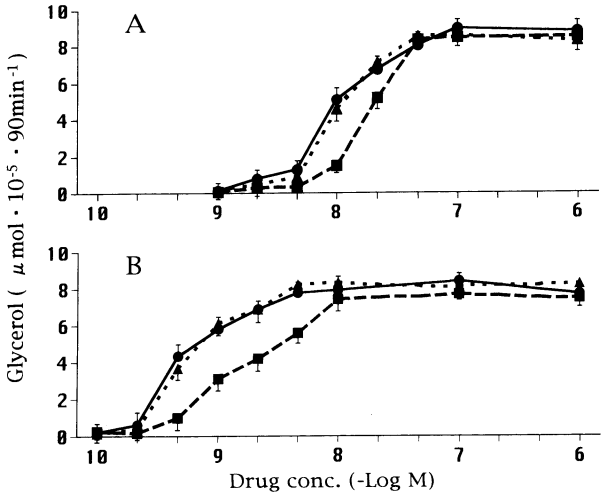

Fig. 1. (A): Isoproterenol-induced glycerol release in isolated subcutaneous (■, N=10), mesenteric (▲, N=10) and epididymal (●, N=10) adipocytes. The results are expressed as the means ± SEM. The lipolytic action of Isop was significantly ($P<0.01$) greater in the epididymal and mesenteric than in the subcutaneous adipocytes in terms of the pD_2 value, but no significant difference was observed between the epididymal and mesenteric adipocytes. (B): CL316,243-induced glycerol release in isolated subcutaneous (■, N=10), mesenteric (▲, N=10) and epididymal (●, N=10) adipocytes. The results are expressed as the means ± SEM. The lipolytic action of CL was significantly ($P<0.01$) greater in the epididymal and mesenteric adipocytes than in the subcutaneous adipocytes in terms of the pD_2 value, but no significant difference was observed between the epididymal and mesenteric adipocytes.

lipolysis at more than 10^{-10}M and showed 100% lipolysis at more than 10^{-8}M in subcutaneous, mesenteric and epididymal adipocytes. The pD_2 value of CL was 8.74 ± 0.05 in subcutaneous, 9.31 ± 0.05 in epididymal and 9.22 ± 0.06 in mesenteric adipocytes. The lipolytic action of CL was significantly ($P<0.01$) greater in the epididymal and mesenteric than in the subcutaneous adipocytes in terms of the pD_2 value, but no significant difference was observed between the epididymal and mesenteric adipocytes.

The lipolytic action of CL was about 10 times higher than that of Isop in terms of the pD_2 value in the three fat sites.

The intrinsic activity (the percentage of maximal response induced by agonist in each experiment) of CL which is related to maximum lipolytic rate of Isop was 90 ± 3% in subcutaneous, 97 ± 4% in mesenteric and 94 ± 4% in epididymal adipocytes. No significant difference was observed among these three fat sites.

The diameter of the adipocytes were 60.2 ± 13.5 μm for subcutaneous, 58.3 ±10.4 μm for mesenteric, and 59.7 ± 15.2 μm for epididymal fat tissues, when the diameters of 100 fat cells were measured in each fat tissue. No significant difference was observed among the three fat sites.

Discussion

Our findings showed that in rats the lipolysis of white adipocytes was induced both by the non-specific β-adrenoceptor agonist, Isoproterenol (Isop), and the β_3-specific adrenoceptor agonist, CL316,243 (CL), and that the lipolytic sensitivity to CL316,243 was about 10 times greater than that to Isop in these epididymal, mesenteric and subcutaneous adipocytes. Regarding the action on the different fat cells, both Isop and CL induced more marked lipolysis in the epididymal and mesenteric adipocytes than in subcutaneous adipocytes in terms of the pD_2 value.

Regarding the relative affinities of Isop and CL316,243 for the various β-adrenergic receptors, it was reported [3] that the β_3-, β_1- and β_2-adrenergic activities (EC_{50}) were 0.013, 0.0015 and 0.0053 for Isop and 0.0030, >100 and 30 for CL316,243, respectively. The selectivity of β_3/β_1 and β_3/β_2 was 0.11 and 0.41 for Isop and >33,000 and 10,000 for CL316,243, suggesting that CL316,243 is a highly specific β_3-adrenoceptor agonist. The pD_2 value and the intrinsic activity which are related to the epididymal fat of rats were reportedly [6, 7] 7.6 and 100% for Isop, 6.8 and 90% for adrenaline, 6.9 and 100% for noradrenaline, 7.0 and 110% for Fenoterol (β_2-agonist), 5.8 and 100% for Salbutamol (β_2-agonist), 8.7 and 100% for BRL37,344 and 5.9 and 40% for CGP12177. The present findings showing that the pD_2 value and the intrinsic activity in epididymal adipocytes were 8.02 ± 0.07 and 100% for Isop and 9.31 ± 0.05 and 94 ± 4% for CL316,243, was more lipolytic than those [6, 7] reported above. Although the difference between our results and other data was not unclear, it may be explained by the body weight of rats used; we used young rats (150 g) in which the size of the

adipocytes does not vary among the regional sites [19, 20], because lipolysis rates are higher in large fat cells than in small fat cells [21, 22], but others used more adult rats (160–250 g) [17, 23–25]. Regarding this point, we also plan to do further examinations with animals.

On the one hand, Simard *et al.* [25] reported that in such an experiment as the determination of lipolytic rates, *in vitro*, preincubation for 15 min is necessary to remove extracellular agents (fatty acids, adenosine, lactate, etc.) that may affect the linearity of lipolytic rates. In this study, preincubation was conducted for 5 min.

The number of β_1-, β_2- and β_3-adrenoceptors in the visceral and the subcutaneous fats of rats have not been reported, although the numbers of β_1- and β_2-adrenoceptors in humans have been reported to be greater in omental white adipocytes than in subcutaneous adipocytes [26]. The m-RNA expression of β_3-adrenoceptors in human white fats has been reported [14] to be highest in the perirenal white adipose tissue (WAT) followed in order by omental WAT and subcutaneous WAT. Our present findings suggested that the number of β_3-adrenoceptors in rats is also greater in visceral adipocytes than in subcutaneous adipocytes, although Sztalryd *et al.* [27] reported that hormone sensitive lipase (HSL) mRNA levels were higher in epididymal and retroperitoneal adipocytes than in subcutaneous adipocytes in rats. Further examinations will therefore be conducted to test this hypothesis by means of the RT-PCR technique. Furthermore, Lönnqvist *et al.* [28] recently reported the existence of a functional β_3-adrenoceptor in humans by showing that CGP12177 (at present the only known selective β_3-adrenoceptor agonist in humans) was more lipolytic in omental than in subcutaneous adipocytes, and the antagonist [CGP20712A (selective β_1-antagonist), ICI118,551 (selective β_2-antagonist)] did not alter the lipolysis induced by CGP12177. Enocksson *et al.* [29] also demonstrated the existence of an *in vivo* functional β_3-adrenoceptor in humans by means of a microdialysis technique. The existence of a functional β_3-adrenoceptor is therefore certain even in humans, although there were some contradictory reports showing that β_3-adrenoceptors are not functional in human adipocytes [24, 30, 31]. The accumulation of visceral fat was shown to be closely associated with diabetes mellitus, hyperlipidemia, hypertension, and arteriosclerosis [8–11]. Our present findings showing that CL is more lipolytic in the mesenteric adipocytes than in subcutaneous adipocytes therefore suggest that CL may be especially useful for the treatment of visceral fat type obesity that complicates these various diseases.

Acknowledgements

We thank the American Cyanamid Company for the gift of CL316,243. This work was supported in part by Grants-in-Aid (No. 06671044 and 07671149) for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by The Smoking Research Foundation, Japan.

References

1. Arch JRS, Ainsworth AT, Cawthorne MA, Piercy V, Sennit M, Thody V, Wilson C, Wilson S (1984) Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature* 309: 163–165.
2. Bloom JD, Dutia MD, Johnson BD, Wissner A, Burns MG, Largis EE, Dolan JA, Claus TH (1992) Disodium (R,R)-5{[2-(3- chlorophenyl)-2-hydroxyethyl]-aminopropyl}-1,3-benzodioxole-2,2-dicarboxylate(CL316,243). A potent β-adrenergic agonist virtually specific for β_3 receptors. A promising antidiabetic and antiobesity agent. *J Med Chem* 35: 3081–3084.
3. Dolan JA, Muenkel HA, Burns MG, Pellegrino SM, Fraser CM, Pietri F, Strosberg AD, Largis EE, Dutia MD, Bloom JD, Bass AS, Tanikella TK, Cobuzzi A, Lai FM, Claus TH (1994) Beta-3 adrenoceptor selectivity of the dioxolane dicarboxylate phenethanolamines. *J Pharmacol Exp Ther* 269: 1000–1006.
4. Yoshida T, Sakane N, Wakabayashi Y, Umekawa T, Kondo M (1994) Anti-obesity and anti-diabetic effects of CL316,243, a highly specific β_3-adrenoceptor agonist, in yellow KK mice. *Life Sci* 54: 491–498.
5. Yoshida T, Sakane N, Wakabayashi Y, Umekawa T, Kondo M (1994) Anti-obesity effect of CL316,243, a highly specific β_3-adrenoceptor agonist, in mice with monosodium-L-glutamate-induced obesity.

Eur J Endocrinol 13: 97–102.
6. Arch JRS, Kaumann AJ (1993) β_3 and atypical β-adrenoceptors. *Med Res Rev* 13: 663–729.
7. Lafontan M, Berlan M (1993) Fat cell adrenergic receptors and the control of white and brown fat cell function. *J Lipid Res* 34: 1057–1091.
8. Kalkhoff RK, Hartz AH, Rupley D, Kissebah AH, Kelber S (1983) Relationship of body fat distribution to blood pressure, carbohydrate tolerance, and plasma lipids in healthy obese women. *J Lab Clin Med* 102: 621–627.
9. Fujioka S, Matsuzawa Y, Tokunaga K, Tarui S (1987) Contribution of intraabdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity. *Metabolism* 36: 54–59.
10. Després JP, Nadeau A, Tremblay A, Ferland M, Moorjani S, Lupien PJ, Thériault G, Pinault S, Bouchard C (1989) Role of deep abdominal fat in the association between regional adipose tissue distribution and glucose tolerance in obese women. *Diabetes* 38: 304–309.
11. Matsuzawa Y, Fujioka S, Tokunaga K, Tarui S (1992) Classification of obesity with respect to morbidity. *Proc Soc Exp Biol Med* 200: 197–201.
12. Leibel RL, Hirsch J (1987) Site- and sex-related differences in adrenoceptor status of human adipose tissue. *J Clin Endocrinol Metab* 64: 1205–1210.
13. Fujioka S, Matsuzawa Y, Tokunaga K, Keno Y, Kobatake T, Tarui S (1991) Treatment of visceral fat obesity. *Int J Obes* 15: 59–65.
14. Krief S, Lönnqvist F, Raimbault S, Baude B, Van Spronsen A, Arner P, Strosberg AD, Ricquier D, Emorine LJ (1993) Tissue distribution of β_3-adrenergic receptor mRNA in man. *J Clin Invest* 91: 344–349.
15. Wilson C, Wilson S, Piercy V, Sennitt MV, Arch JRS (1984) The rat lipolytic β-adrenoceptor: Studies using novel β-adrenoceptor agonists. *Eur J Pharmacol* 100: 309–319.
16. Sugasawa T, Matsuzaki M, Morooka S, Foignant N, Blin N (1992) *In vitro* study of a novel atypical β-adrenoceptor agonist, SM-11044. *Eur J Pharmacol* 216: 207–215.
17. Rodbell M (1964) Metabolism of isolated fat cells. *J Biol Chem* 239: 375–380.
18. Eggstein M, Kreutz FH (1996) Eine neue bestimmung der neutralfette im blutserum und gewebe. *Klin Wschr* 5: 262–267.
19. Fried SK, Lavau M, Pi-Sunyer FX (1982) Variations in glucose metabolism by fat cells from three adipose depots of the rat. *Metabolism* 31: 876–883.
20. Santos RF, Sztalryd C, Reaven G (1991) Effect of anatomical site on insulin action and insulin receptor phosphorylation in isolated rat adipocytes. *Int J Obes* 15: 755–762.
21. Hartman AD, Christ DW (1978) Effect of cell size, age and anatomical location on the lipolytic response of adipocytes. *Life Sci* 22: 1087–1096.
22. Gruen R, Kava R, Greenwood MRC (1980) Development of basal lipolysis and fat cell size in the epididymal fat pad of normal rats. *Metabolism* 29: 246–253.
23. Hollenga C, Hass M, Deinum JT, Zaagsma J (1990) Discrepancies in lipolytic activities induced by β-adrenoceptor agonists in human and rat adipocytes. *Horm Metabol Res.* 22: 17–21.
24. Langin D, Portillo MP, Saulnier-Blache JS, Lafontan M (1991) Coexistence of three β-adrenoceptor subtypes in white fat cells of various mammalian species. *Eur J Pharmacol* 199: 291–301.
25. Simard PM, Atgié C, Mauriège P, D'Allaire F, Bukowiecki LJ (1994) Comparison of the lipolytic effects of norepinephrine and BRL37344 in rat brown and white adipocytes. *Obes Res* 2: 424–431.
26. Hellmér J, Marcus C, Sonnenfeld T, Arner P (1992) Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. *J Clin Endocrinol Metab* 75: 15–20.
27. Sztalryd C, Kraemer FB (1994) Differences in hormone-sensitive lipase expression in white adipose tissue from various anatomic locations of the rat. *Metabolism* 43: 241–247.
28. Lönnqvist F, Krief S, Strosberg AD, Nyberg B, Emorine LJ, Arner P (1993) Evidence for a functional β_3-adrenoceptor in man. *Br J Pharmacol* 110: 929–936.
29. Enocksson S, Shimizu M, Lönnqvist F, Nordenström J, Arner P (1995) Demonstration of an *in vivo* functional β_3-adrenoceptor in man. *J Clin Invest* 95: 2239–2245.
30. Hollenga C, Brouwer F, Zaagsma J (1991) Differences in functional cyclic AMP compartments mediating lipolysis by isoprenaline and BRL37344 in four adipocyte types. *Eur J Pharmacol* 200: 325–330.
31. Van Liefde I, Van Ermen A, Vauquelin G (1994) No functional atypical β-adrenergic receptors in human omental adipocytes. *Life Sci* 54: 209–214.