An assay for beta-adrenergic receptors in isolated human fat cells

Peter Engfeldt, Peter Arner, Hans Wahrenberg, and Jan Östman

Department of Medicine and the Research Center, Huddinge Hospital, Karolinska Institute, S-141 86 Huddinge, Sweden

Abstract The beta-adrenergic receptors have been characterized in isolated human adipocytes using a potent beta-adrenergic antagonist (−)-[^{3}H]dihydroalprenolol. Binding of (−)-[^{3}H]dihydroalprenolol to isolated fat cells was stereospecific and saturable, the maximum number of binding sites calculated being 7.8 ± 2.2 pmol of bound ligand/10^7 cells, corresponding to 450,000 binding sites/cell. The dissociation constant was estimated to be 2.7 ± 1.1 nM. The results with competition–inhibition experiments using beta-adrenergic agonists and antagonists indicated that the binding sites in isolated adipocytes were predominantly of the beta$_1$-subtype; about 80% of the receptors were of this type. With the present method, specific beta-adrenergic receptor number and affinity in isolated human adipocytes could be determined in about 1 g of human adipose tissue.—**Engfeldt, P., P. Arner, H. Wahrenberg, and J. Östman.** An assay for beta-adrenergic receptors in isolated human fat cells. *J. Lipid Res.* 1982. **23:** 715–719.

Supplementary key words catecholamines • radioligand • adipose tissue

The catecholamines are potent stimulators and inhibitors of lipid mobilization in human adipose tissue (1, 2). Since these sympathoadrenal neurotransmitters have a role in certain common disorders such as diabetes mellitus (3), hypertension (4), and hyperthyroidism (5), knowledge about their biological activity is of considerable interest. Recently, the adrenergic receptors in membranes from human adipocytes have been identified (6, 7). To enable investigations of the beta$_1$-adrenoceptors in fat cells from a certain individual, the present assay of beta-adrenoceptors with the radioligand (−)-[^{3}H]dihydroalprenolol was worked out for isolated fat cells available by simple biopsy procedure. This method also avoids the alterations in hormonal binding sites, which may occur during preparation of membrane fractions (8). Studies on the identification and characteristics of the beta-adrenergic receptors in intact human fat cells are reported and the findings are discussed and compared with previous findings in isolated rat fat cells (9, 10).

MATERIALS AND METHODS

Subcutaneous adipose tissue was obtained at operations from patients who were operated for gall-bladder disease or gastric ulcer. They had fasted overnight. None had jaundice, metabolic disorder, or malignant disease. No attempt was made to select the patients on the basis of age, sex, or body weight. General anesthesia was induced with a short-acting barbiturate and maintained by Leptanal® (Leo, Sweden). Saline was infused intravenously prior to the biopsy. The specimen of adipose tissue was taken at the start of the operation and brought to the laboratory in saline. The study was approved by the Ethical Committee of the Karolinska Institute.

Isolation of fat cells

The adipose tissue specimen was cut into fragments, each weighing about 10 mg. Fat cells were isolated from the stroma by incubation with 2.5 mg of collagenase for 60 min in 5 ml of Krebs-Ringer bicarbonate (KRB) buffer, containing 40 mg of bovine serum albumin per ml, by the method of Rodbell (11). The adipocytes were washed three times in KRB buffer and aggregated material was removed by repeated filtration through silk cloth. The yield was 1 ml of packed fat cells per g of adipose tissue.

Binding assay

Isolated fat cells were incubated with different concentrations of (−)-[^{3}H]dihydroalprenolol (as specified in figure legends) in a total volume of 1 ml of incubation buffer containing 125 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 25 mM Tris–HCl, 5 mg/ml albumin, and 0.1 mg/ml ascorbic acid (pH 7.4).

Specific binding was not changed by varying the concentration of albumin in the incubation buffer from 1 to 40 mg/ml. In competition experiments, unlabeled compounds were added from fresh stock solutions. The

incubation was initiated by the addition of 300-μl aliquots of fat cells suspended in the incubation buffer. After 15 min of incubation at 37°C, the incubations were terminated by adding 8 ml of wash buffer (154 mM NaCl, 4°C) to each tube and the contents in the tubes were then rapidly filtered under vacuum through a single Whatman GF/C filter. The filters were then washed with two 8-ml aliquots of wash buffer. This procedure was found to reduce the non-specific binding without affecting the specific binding. The filters were dried and taken for determination of radioactivity using a scintillation mixture of toluene containing 2,5-diphenyloxazole (5 g/l) and 1,4-bis-2-(4 methyl-5-phenyl-oxasolyl)-benzene (0.3 g/l). Assay tubes were run in triplicate.

In each experiment, non-specific binding to fat cells was determined by measuring the amount of radioactivity retained on filters from parallel incubations containing 0.5 μM unlabeled (±) propranolol in addition to the labeled ligand.

The binding values reported refer to specific binding determined by subtracting the non-specific binding from the total counts found at each concentration of the radioligand. Binding data were analyzed according to Scatchard (12) and Hofstee (13).

The coefficient of variation was about 5%.

Determination of fat cell number

The mean fat cell triglyceride weight was determined as described in detail previously (14). The number of fat cells incubated was obtained by dividing the lipid weight of the incubated fat cells by the mean fat cell lipid weight.

Chemicals

Bovine serum albumin, fraction V, was purchased from Armour Pharmaceutical Company, Eastbourne, England. Collagenase prepared from *Clostridium histolyticum* was Sigma type I. (−)-[^{3}H]Dihydroalprenolol (sp act 44.9 Ci/mmol) was from the New England Nuclear Corporation, Boston. (±) Propranolol, (−) isopropyl noradrenaline, (−) noradrenaline, and (−) adrenaline were from Sigma, Saint Louis. (+) Propranolol and (−) propranolol were from ICI, Macclesfield, England. Metoprolol and butoxamine were gifts from Hässle, Gothenburg, Sweden and phentolamine was a gift from Ciba-Geigy, Gothenburg, Sweden. All other chemicals were of the highest purity commercially available.

Statistical analyses

Each experiment was repeated three to four times to assure consistency. The values given are the mean and the standard deviation. Linear regression analysis was performed by the method of least squares. Curvilinear binding data were transformed to two linear regression lines using a computerized two-site model (15). All calculations were made on a Wang 2200 computer.

RESULTS

The non-specific binding was determined with 0.5 μM (±) propranolol. This concentration of (±) propranolol was found to be about 100 times its dissociation constant value (K_D), which is considered to be the optimal concentration for the determination of non-specific binding (16). It was also found that isopropyl noradrenaline, in a concentration of about 100 times its K_D, could be used for determination of the non-specific binding. However, (±) propranolol was preferred since it is more stable than isopropyl noradrenaline against oxidation. The non-specific binding was directly proportional to the total (−)-[^{3}H]dihydroalprenolol concentration in the media, 40% of total binding at 0.5 nM (−)-[^{3}H]dihydroalprenolol and 80% at 10 nM.

In separate experiments it was noted that the addition of phentolamine in concentrations from 10^{-6} to 10^{-3} M decreased the total as well as the specific binding to fat cells (data not shown). Specific binding was proportional to cell number over the range of 1 to 5×10^4 cells/ml and therefore a concentration of 3×10^4 cells/ml was chosen in the further experiments; this amount corresponds to about 25 μl of packed cells per incubation tube.

The kinetics of (−)-[^{3}H]dihydroalprenolol binding to fat cells is shown in **Fig. 1.** Binding was quite rapid; steady state was achieved within 5 min. Dissociation of bound (−)-[^{3}H]dihydroalprenolol was monitored by assaying the amount of bound radioligand remaining following the addition of 0.5 μM (±) propranolol to fat

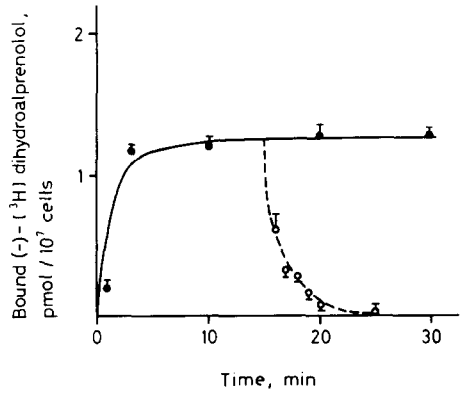

Fig. 1. Time course of specific binding of (−)-[^{3}H]dihydroalprenolol to isolated human adipocytes and dissociation of (−)-[^{3}H]-dihydroalprenolol from human adipocytes. (−)-[^{3}H]Dihydroalprenolol (2 nM) was added to isolated adipocytes and specific binding was determined at the indicated times (solid line). At 15 min, (±) propranolol was added (final concentration 0.5 μM) and specific bound (−)-[^{3}H]dihydroalprenolol was determined at indicated times (broken line) (n = 3).

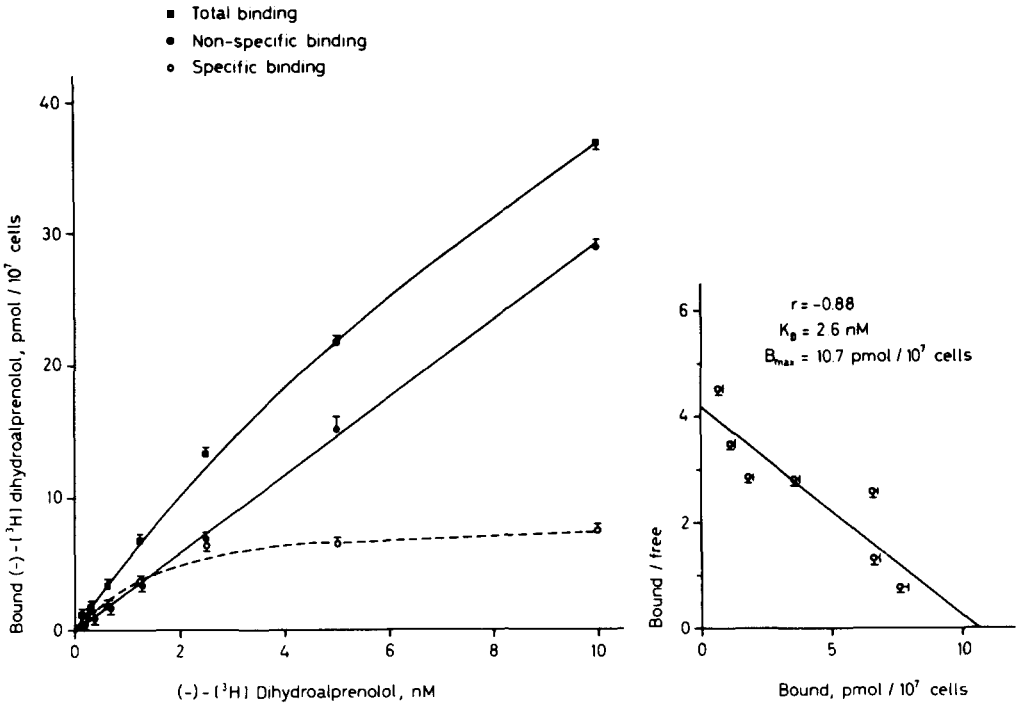

Fig. 2. (−)-[^{3}H]Dihydroalprenolol binding to isolated human adipocytes as a function of radioligand concentration. Incubation time was 15 min. Left: total (filled squares), non-specific (filled circles), and specific (open circles) binding as a function of total concentrations of (−)-[^{3}H]dihydroalprenolol. The values are the mean and the standard deviations (n = 3). Right: Scatchard plot of specific (−)-[^{3}H]dihydroalprenolol binding to isolated adipocytes. The results obtained from the left panel of the figure were calculated according to Scatchard (12).

cells that had already attained a steady state level of binding with 2 nM (−)-[^{3}H]dihydroalprenolol. (−)-[^{3}H]Dihydroalprenolol dissociated from the fat cells with a half-life of about 60 sec and complete dissociation occurred within 10 min.

Incubation of human fat cells with increasing concentrations of (−)-[^{3}H]dihydroalprenolol (0.5–10 nM) showed that specific binding is a saturable process (**Fig. 2**). From the figure it can be seen that half maximal binding occurred at a concentration of about 1 nM ligand. Saturation was observed when about 8 pmol ligand were bound/10^7 cells. From five different experiments, the dissociation constant (K_D) was calculated to be 2.7 ± 0.5 nM and the maximum number of binding sites (B_{max}) was estimated to be 7.8 ± 1.0 pmol (−)-[^{3}H]dihydroalprenolol bound/10^7 cells, which corresponds to 450,000 binding sites/cell. The Scatchard plot (12) was linear with a correlation coefficient of 0.88, which indicates a homogenous population of receptors. It is observed in Fig. 2 that an accurate Scatchard plot could be constructed with binding data from a saturation experiment using seven different concentrations of the radioligand. As mentioned previously, total and non-specific binding were determined in triplicate, respectively, with 25 μl of packed isolated fat cells per incubation tube. Thus, B_{max} and K_D could be determined on 1 ml of packed fat cells, which is equivalent to 1 g of adipose tissue.

The influence of various beta-adrenergic agonists to compete for (−)-[^{3}H]dihydroalprenolol binding to fat cells was examined (**Fig. 3**). The beta-adrenergic agonists competed for (−)-[^{3}H]dihydroalprenolol binding sites with the following order of potency: (−) isopropyl noradrenaline > (−) noradrenalin ⩾ (−) adrenaline. This order of potency would indicate a beta$_1$ type of adrenergic

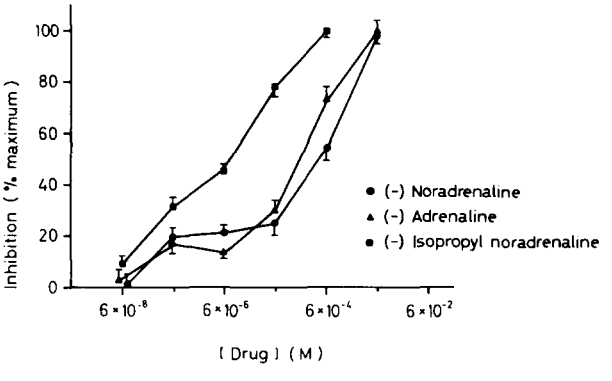

Fig. 3. Inhibition of specific (−)-[^{3}H]dihydroalprenolol binding to isolated human adipocytes by adrenergic agonists. Isolated adipocytes were incubated with (−)-[^{3}H]dihydroalprenolol (2 nM) in the presence or absence of the indicated concentrations of various competitors. Incubation time was 15 min (n = 3).

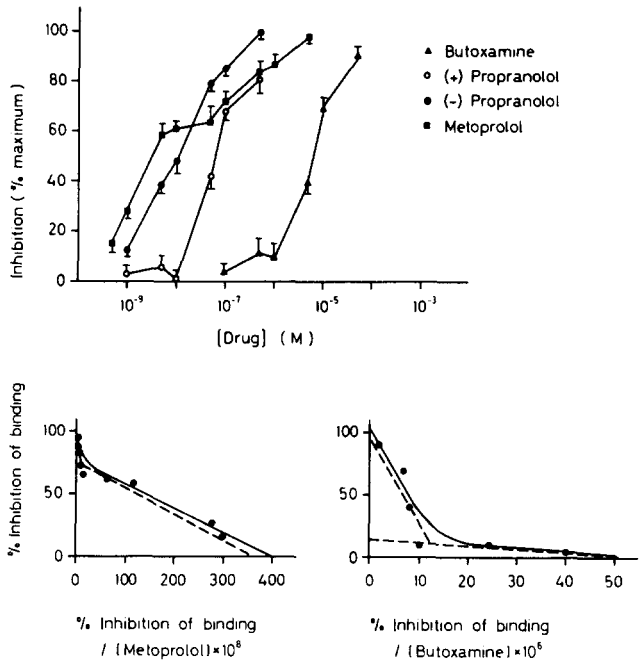

Fig. 4. Inhibition of specific (−)-[^{3}H]dihydroalprenolol binding to isolated human adipocytes by adrenergic antagonists. Upper: isolated adipocytes were incubated with (−)-[^{3}H]dihydroalprenolol (2 nM) in the presence or absence of the indicated concentrations of various competitors. Incubation time was 15 min (n = 3). Lower: Hofstee plots of metoprolol and butoxamine binding to isolated human adipocytes (values obtained from the experiments in the upper panel).

receptor (17). In order to further classify the beta-receptor of isolated human adipocytes, competition–inhibition experiments with beta-adrenergic antagonists were performed (**Fig. 4**). When the (−) and (+) stereoisomers of propranolol were compared, half maximal inhibition of (−)-[^{3}H]dihydroalprenolol binding was achieved with 10 nM (−) propranolol, whereas a 10-fold higher concentration of (+) propranolol was required to elicit the same level of inhibition. This indicates that (−)-[^{3}H]dihydroalprenolol binding is stereospecific. Metoprolol, a specific beta$_1$-antagonist, was approximately 1000 times more effective than butoxamine, a specific beta$_2$-antagonist. Half maximum inhibition of (−)-[^{3}H]dihydroalprenolol binding was achieved with approximately 4 nM metoprolol and 10 μM butoxamine. These data indicate that the beta-receptor of isolated human adipocytes is predominantly of the beta$_1$-subtype. However, data of the competition experiments with metoprolol and butoxamine can be plotted according to Hofstee (13) in order to estimate the proportions of beta$_1$- and beta$_2$-receptors. Although, it has been suggested (18) that at least 15 different concentrations of a competing ligand should be used to precisely calculate the proportions of different subtypes of receptors, the present data in Fig. 4 could be used to get an estimate of the proportion of the receptor subtypes. It is observed in Fig. 4 that curvilinear Hofstee plots (13) were obtained with metoprolol and butoxamine, which is indicative of the existence of two subtypes of beta-receptors. These plots were dissolved into two straight lines using a computerized two-site model (15). It was then found, using metoprolol data, that about 75% of the receptors were of beta$_1$-subtype. Using butoxamine data, the proportion of beta$_1$-receptors was approximately 85%.

DISCUSSION

Although the present method using the radioligand (−)-[^{3}H]dihydroalprenolol for investigations of the beta-adrenergic receptors on isolated fat cells is basically similar to previous methods (9, 10) worked out for rat fat cells, significant differences in methodology and results exist.

In the present study, saturation was obtained at about 10 nM (−)-[^{3}H]dihydroalprenolol, whereas in rat fat cells no saturation was obtained by a concentration of up to 100 nM of this radioligand. A large, unspecific and propranolol-sensitive binding was found in rat fat cells and this was eliminated by the addition of a high concentration of phentolamine to the assay system. This is in contrast to our findings with human fat cells. The reason for this discrepancy remains unclear. Scatchard plots of steady state (−)-[^{3}H]dihydroalprenolol binding to human fat cells were linear, but were curvilinear with an upward concavity in rat fat cells. The findings in the rat cells could be explained either by several populations of binding sites or by negative cooperative interaction between the receptors. In the human fat cells, it seems that there is a homogenous population of receptors and about 80% of these receptors appears to be of the beta$_1$-subtype. The value obtained here for the dissociation constant (2.7 nM) is lower than that reported from studies with rat fat cells (8–10, 19) but is in the same range as that reported for hamster brown adipocytes (20) and broken membranes of human fat cells (6, 7).

However, there are also similarities between human and rat beta-adrenergic receptors in fat cells. Stereospecificity is demonstrated in fat cells from both species. The number of beta-receptors per fat cell appears also to be similar in man and rat (9), about 400,000 sites/cell.

The total amount of subcutaneous adipose tissue needed to perform an experiment from which receptor number and affinity could be determined was 1 g. Since this small amount of tissue is easily obtained from subcutaneous fat depots by surgical biopsy (21), the present method for determination of beta-adrenergic receptors in isolated human adipocytes can be used in clinical studies on non-obese subjects.

The authors are grateful to Dr. Craig Malbon for the generous gift of a special device for filtration and to Drs. Jan Svartengren and Barbara Cannon for valuable discussion. This investigation was supported by grants from the Swedish Diabetes Association, the Osterman, the Groschinsky, and the Amundson Foundations.

Manuscript received 22 May 1981 and in revised form 22 December 1981.

REFERENCES

1. Björntorp, P., and J. Östman. 1971. Human adipose tissue dynamics and regulation. *Adv. Metab. Disord.* **5:** 227–327.
2. Burns, T. W., B. E. Terry, P. E. Langley, and G. A. Robison. 1980. Role of cyclic AMP in human adipose tissue lipolysis. *Adv. Cyclic Nucleotide Res.* **12:** 329–341.
3. Christensen, N. J. 1979. The role of catecholamines in clinical medicine. *Acta Med. Scand. Suppl.* **624:** 9–18.
4. De Champlain, J. 1977. The sympathetic system in hypertension. *Clin. Endocrinol. Metab.* **6:** 633–655.
5. Landsberg, L. 1977. Catecholamines and hyperthyroidism. *Clin. Endocrinol. Metab.* **6:** 697–718.
6. Burns, T. W., P. E. Langley, B. E. Terry, D. B. Bylund, B. B. Hoffman, M. D. Tharp, R. J. Lefkowitz, J. A. Garcia-Sainz, and J. N. Fain. 1981. Pharmacological characterizations of adrenergic receptors in human adipocytes. *J. Clin. Invest.* **67:** 467–475.
7. Jacobsson, B., G. Vaugelin, C. Wesslau, U. Smith, and A. D. Strosberg. 1981. Distinction between two subpopulations of beta$_1$-adrenergic receptors in human adipose cells. *Eur. J. Biochem.* **114:** 349–354.
8. Malbon, C. C., F. J. Moreno, R. J. Cabelli, and J. N. Fain. 1978. Fat cell adenylate cyclase and beta-adrenergic receptors in altered thyroid status. *J. Biol. Chem.* **253:** 671–678.
9. Cabelli, R. J., and C. C. Malbon. 1979. Characterization of (−)-[^{3}H]dihydroalprenolol binding sites on isolated rat fat cells. *J. Biol. Chem.* **254:** 8903–8908.
10. Goswami, A., and I. N. Rosenberg. 1978. Thyroid hormone modulation of epinephrine-induced lipolysis in rat adipocytes: a possible role of calcium. *Endocrinology.* **103:** 2223–2233.
11. Rodbell, M. 1964. Metabolism of isolated fat cells. 1. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375–380.
12. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51:** 660–672.
13. Hofstee, B. H. J. 1952. On the evaluation of the constants V_m and K_m in enzyme reactions. *Science.* **116:** 329–331.
14. Östman, J., L. Backman, and D. Hallberg. 1975. Cell size and antilipolytic effect of insulin in human subcutaneous adipose tissue. *Diabetologia.* **11:** 159–164.
15. Feldman, H. A. 1972. Mathematical theory of complex ligand-binding systems at equilibrium: some methods for parameter fitting. *Anal. Biochem.* **48:** 317–338.
16. Weiland, G. A., and P. B. Molinoff. 1981. Quantitative analysis of drug-receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.* **29:** 313–330.
17. Lands, A. M., A. Arnold, J. P. McAuliff, F. P. Ludveno, and T. G. Brown. 1967. Differentiation of receptor systems activated by sympathomimetic amines. *Nature.* **214:** 597–598.
18. Molinoff, P. B., B. B. Wolfe, and G. A. Weiland. 1981. Quantitative analysis of drug-receptor interactions. II. Determination of the properties of receptor subtypes. *Life Sci.* **29:** 427–443.
19. Williams, L. T., J. Leonard, and R. J. Lefkowitz. 1976. Adipocyte beta-adrenergic receptors. Identification and subcellular localization by (−)-[^{3}H]dihydroalprenolol binding. *J. Biol. Chem.* **251:** 3096–3104.
20. Svoboda, P., J. Svartengren, M. Snochowski, J. Hovstek, and B. Cannon. 1979. High number of high-affinity binding sites for (−)-[^{3}H]dihydroalprenolol on isolated hamster brown fat-cells. A study of the beta-adrenergic receptors. *Eur. J. Biochem.* **102:** 203–210.
21. Arner, P., O. Arner, and J. Östman. 1973. The effect of local anaesthetic agents on lipolysis by human adipose tissue. *Life Sci.* **13:** 161–169.