Age-related changes in adrenergic α_1, α_2, and β receptors of rat white fat cell membranes: an analysis using [^{3}H]bunazosin as a novel ligand for the α_1 adrenoceptor

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Abstract Age-related changes in α_1-, α_2-, and β-catecholamine receptors on membranes of rat epididymal fat cells were investigated. Both young (6 weeks old, weight about 190 g) and aged (20 weeks old, weight about 490 g) Sprague-Dawley male rats were used. For the α_1-adrenoceptor binding experiment, we developed a novel analytical method using the hydrophilic α_1-receptor selective antagonist, [^{3}H]bunazosin. The binding of [^{3}H]bunazosin to its binding sites was rapid, reversible, saturable, and stereospecific. Scatchard binding analysis showed a single class of binding site. The sites were characterized as α_1-adrenoceptors by inhibition experiments using various agonists and antagonists. The number of maximum binding sites (B_{max}) of α_1-receptor binding was 37.0 ± 6.5 (young) versus 24.0 ± 3.2 (aged) fmol/mg protein ($P < 0.01$). [^{3}H]Rauwolscine and [^{3}H]CGP-12177 were used for α_2- and β-receptor binding, respectively. In α_2-receptor detection using [^{3}H]rauwolscine as a ligand, B_{max} increased markedly from 19.8 ± 4.9 to 86.2 ± 19.5 fmol/mg protein ($P < 0.01$). In contrast, B_{max} for β-receptor decreased from 69.7 ± 9.7 to 45.4 ± 13.9 fmol/mg protein with increasing rat age ($P < 0.05$). K_d showed no change in each of the binding experiments between young and aged rats. The cell volume increased from 0.07 ± 0.02 to 0.15 ± 0.06 nl. It is implied that anti-lipolytic activity strengthened on the whole mainly with the marked increase of α_2-receptor number and decrease of β-receptor number. Furthermore, [^{3}H]bunazosin was demonstrated to be a new suitable radioligand for the detection of the α_1-adrenoceptor of rat white fat cell membranes. —**Kobatake, T., Y. Watanabe, Y. Matsuzawa, K. Tokunaga, S. Fujioka, T. Kawamoto, Y. Keno, S. Tarui, and H. Yoshida.** Age-related changes in adrenergic α_1, α_2, and β receptors of rat white fat cell membranes: an analysis using [^{3}H]bunazosin as a novel ligand for the α_1 adrenoceptor. *J. Lipid Res.* 1991. **32:** 191–196.

Supplementary key words adipocytes • [^{3}H]bunazosin • [^{3}H]rauwolscine • [^{3}H]CGP-12177 • rat white adipose tissue • aging • cell size

It is generally agreed that catecholamines exert their lipolytic action by stimulating β-adrenoceptors located on the adipocyte membranes and activating adenylate cyclase. On the other hand, catecholamines also perform an anti-lipolytic role by stimulating α_2-adrenoceptors and inhibiting adenylate cyclase (1).

The existence of α_1-adrenoceptors, which could not be shown owing to the impossibility of direct binding of [^{3}H]prazosin (2) on rat white fat cell membranes, has been suggested indirectly by the demonstration of responses to α_1-adrenoceptor agonists such as phosphatidylinositol turnover or Ca^{2+} mobilization (3), although their physiological roles in white fat cells have not been clarified.

The age-related changes occurring in lipolytic function via these receptors have been investigated by some researchers, and opposite data have been reported indicating that β-adrenoceptor density in rat adipocytes decreases with age (4) or that it does not show any change with age (5). With regard to α_2-adrenoceptors, observations in human subjects have also been reported (6, 7). However, there are no distinct opinions about age-related changes in either α_1-, α_2, or β-adrenoceptors.

In the present study, in which young and aged obese Sprague-Dawley (SD) rats were used, we examined the age-related changes in α_1-, α_2-, and β-adrenoceptors of epididymal white fat cell membranes using the ^{3}H-labeled radioligand binding technique. In particular, α_1-adrenergic receptors on white fat cell membranes of rat have not been previously detected by direct binding. Therefore, the aim of the first part of our investigation

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was to demonstrate the usefulness of the new radioligand, [^{3}H]bunazosin, for direct binding of α_1-adrenoceptors on the white fat cell membranes from rats.

MATERIALS AND METHODS

Materials

[^{3}H]CGP-12177 (1.48 TBq/mmol = 40 Ci/mmol), [^{3}H]rauwolscine (2.77 TBq/mmol = 75 Ci/mmol), and [^{3}H]prazosin (2.64 TBq/mmol = 72 Ci/mmol) were purchased from Amersham, Arlington Heights, IL; [^{3}H]bunazosin (2.57 TBq/mmol = 70 Ci/mmol) was obtained from Eizai Co. Ltd., Japan. Yohimbine hydrochloride and l-norepinephrine bitartrate were purchased from Nacalai Tesque, Inc., Japan. Bovine serum albumin (fraction V), l-isoproterenol hydrochloride and (±) propranolol hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO. The following drugs were purchased from the respective companies: l-epinephrine, Wako Pure Chemical Industries, Ltd., Japan; d-epinephrine, ICN Nutritional Biochemicals, Irvine, CA; prazosin hydrochloride, Taito-Pfizer, Japan; collagenase class I, Worthington, Freehold, NJ. All other chemicals used were of reagent grade or better. Male Sprague-Dawley rats purchased from Charles River were housed in a climate-regulated vivarium with a 12-h light/12-h dark cycle and fed ad libitum with a standard diet with free access to drinking water. The weights of two representative groups of rats at the ages of 6 and 20 weeks were 190 ± 15 g and 490 ± 45 g (n = 30 and 10) (mean ± SD), respectively.

Isolation of white fat cells and preparation of fat cell crude membranes of rats

Epididymal fat pads were quickly removed after rats were killed by decapitation, and processed immediately to obtain crude membrane preparations. The mean volume of epididymal fat cells was significantly larger in the aged obese SD rats (20 weeks) than in the young SD rats (6 weeks) (0.15 ± 0.06 nl vs. 0.07 ± 0.02 nl, $P < 0.001$). Isolated fat cells were prepared according to the method of Rodbell (8) with a slight modification (9). The fat pads were put into polyethylene vials each containing 5 ml of Krebs-Ringer-phosphate-HEPES (KRPH) buffer (130 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.24 mM $MgSO_4$, 2.5 mM Na_2PO_4, and 10 mM HEPES) with 3.5% bovine serum albumin, 1.0 mg/ml collagenase, and 0.6 mM D-glucose at pH 7.4, and then minced. The vials were shaken at 140 cycles/min at 37°C for about 60 min. The cell suspension was then passed through a nylon filter (202 μm, Nitex) and washed three times with KRPH buffer containing 1% albumin and 5.5 mM glucose, pH 7.4, at 37°C. The fat cells were then suspended and incubated in KRPH buffer with 1% BSA and 5.5 mM glucose, pH 7.4, at 37°C for 20 min in order to repair cell surface damage (10). At the end of the incubation period, isolated fat cells were suspended in 50 mM ice-cold Tris-HCl buffer, pH 7.4, and homogenized with a Physcotron (setting 10, 60 sec, 2 times). The homogenate was immediately centrifuged at 11,000 *g* for 20 min at 4°C. The floating fat cake and infranatant were discarded and the remaining pellet was redispersed in the same buffer and recentrifuged under similar conditions. At the end of the washing procedure, the final pellets (crude adipocyte membranes) were resuspended in the same buffer, resulting in a suspension containing about 1.5 mg of membrane protein/ml, and immediately frozen. The membrane preparation was stored at −80°C and used for the binding assay within 1–2 weeks. Protein concentration was determined by the method of Lowry et al. (11), using bovine serum albumin as a standard. Adipocyte size was measured (12) and the mean cell volume was calculated.

Binding assays

Thawed frozen adipocyte membranes were rehomogenized, and binding assays were performed with [^{3}H]CGP-12177 (13), [^{3}H]rauwolscine, and [^{3}H]bunazosin in a final volume of 1 ml with 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$. Membrane protein contents were 50–100 μg/tube. Nonspecific binding was evaluated in the presence of 10 μM (±) propranolol, 10 μM yohimbine, and 10 μM prazosin for binding of the ^{3}H-labeled radioligand CGP-12177, rauwolscine, and bunazosin, respectively. Incubations were carried out at 25°C in a water bath for 20 min under constant shaking. At the end of the incubation, each suspension was diluted in 4.5 ml of ice-cold buffer and filtered through a Whatman GF/F glass fiber filter. The filter was washed twice with 4.5 ml of ice-cold incubation buffer, dried, and then placed in a minivial containing 5 ml of liquid scintillation mixture (Clear-sol I, Nacalai Tesque, Japan) and counted in a liquid scintillation spectrometer at an efficiency of about 40%. Specific binding was defined as the total binding minus the nonspecific binding. Saturation curves for ^{3}H-labeled radioligands were analyzed by Scatchard plots to determine the equilibrium dissociation constant (K_d) and the number of maximum binding sites (B_{max}). The K_i values for nonradioactive agonists and antagonists were calculated by the method of Cheng and Prosoff (14) from the drug concentrations causing 50% displacement of [^{3}H]bunazosin binding.

Data presentation

All experiments were performed in duplicate and repeated 4–6 times. Data are presented as means ± SD. The significance of differences was tested by Student's *t* test.

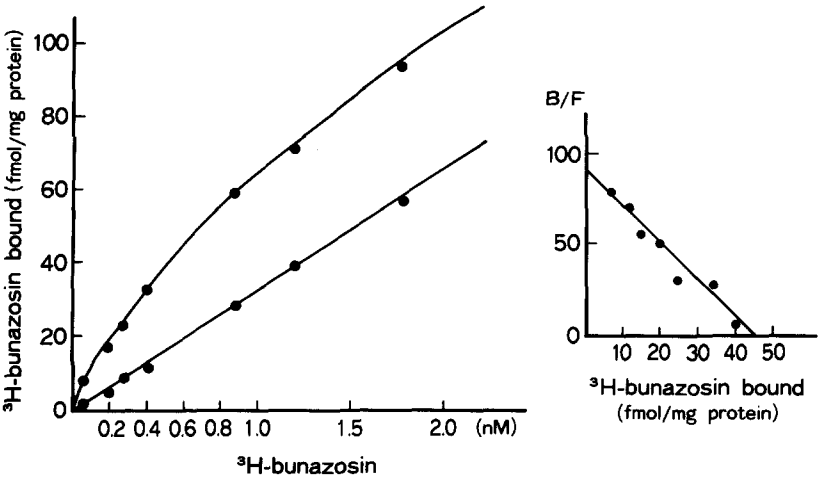


Fig. 1. Specific binding of [³H]bunazosin to young rat epididymal fat cell membranes from one representative experiment. Saturation curves and Scatchard plot of specific binding (inserted). Rat fat cell membranes were incubated with the indicated concentrations of [³H]bunazosin for 20 min at 25°C and specific binding was determined as described in Materials and Methods. Nonspecific binding was defined with 10 μM unlabeled prazosin.

RESULTS

Binding of [³H]bunazosin to rat white fat cell membranes

Specific binding was directly proportional to the membrane protein concentration at the concentrations of ligand and membrane (50–100 μg/tube) used (data not shown).

Saturation binding experiments and Scatchard analysis revealed that the binding consisted of a single class with a dissociation constant (K_d) of 0.58 ± 0.03 nM and B_{max} of 37.0 ± 6.5 fmol/mg protein (n = 6). The data from one representative experiment is shown in **Fig. 1**. The binding was monophasic.

Kinetics of [³H]bunazosin specific binding are shown in **Fig. 2**. The association of [³H]bunazosin with its binding sites was rapid, with equilibrium being reached within 10 min and maintained for at least 35 min at 25°C. The dissociation was determined at 25°C by incubating adipocyte membranes with [³H]bunazosin to equilibrium then adding 10 μM prazosin and measuring the residual specific binding at subsequent time intervals. The bimolecular rate constant (K_1) for this association process was calculated according to Williams, Mullikin, and Lefkowitz (15), and averaged 0.6136 min^{-1} nM^{-1}. The rate of dissociation of [³H]bunazosin binding was examined by incubating [³H]bunazosin and the membranes to equilibrium at 25°C and then adding 10 μM prazosin to prevent rebinding of the dissociated [³H]bunazosin. The rate constant for the dissociation (K_{-1}) of [³H]bunazosin averaged 0.36938 min^{-1}. The dissociation constant calculated from the rate of (K_{-1}/K_1) was 0.602 nM which is in good agreement with the Scatchard analysis of specific binding isotherms determined from equilibrium binding experiments.

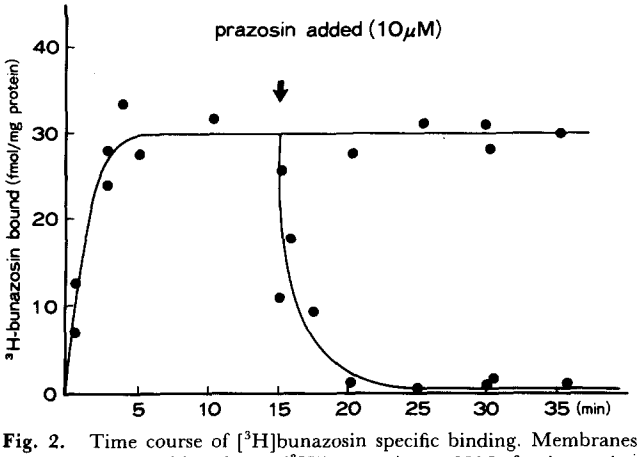


Fig. 2. Time course of [³H]bunazosin specific binding. Membranes were incubated with 2.0 nM [³H]bunazosin at 25°C for increasing periods of time and the amount of specific binding was measured. At the arrow, prazosin was added to the incubation mixture at a final concentration of 10 μM.

Competition studies with some antagonists (**Fig. 3**) and agonists (**Fig. 4**) were performed. They revealed that the relative order of potency of the various antagonists in displacing [³H]bunazosin from its binding site was prazosin > yohimbine > propranolol > atropine, with respective K_i values (M) of 7.5×10^{-10}, 7.5×10^{-6}, 2.5×10^{-5}, and 7.5×10^{-5}. Various adrenergic agonists also competed with [³H]bunazosin binding in the following order of potency; l-norepinephrine > l-epinephrine > d-epinephrine > isoproterenol, with respective K_i values (M) of 1×10^{-7}, 1×10^{-6}, 1×10^{-5}, and 1×10^{-3}. In other words, the l-stereoisomer of epinephrine was more potent than its d-analog in competing for [³H]bunazosin binding sites.

The nonspecific binding was 48.1% of total binding at a free ligand concentration of 0.7 nM for [³H]bunazosin. When [³H]prazosin, which has been used as an α_1-selective antagonist in other types of cells, was used in this experiment as ligand, effective specific binding was not obtained because of its high nonspecific binding.

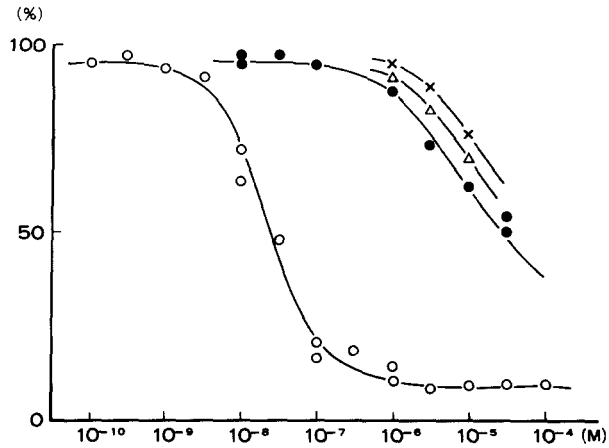


Fig. 3. Inhibition of [^{3}H]bunazosin binding using various antagonists. Rat epididymal fat cell membranes (50–100 μg/ml) were incubated for 20 min at 25°C with [^{3}H]bunazosin (1 nM) and the indicated concentrations of prazosin (○), yohimbine (●), propranolol (△), and atropine (×). Binding assays were performed as described in Materials and Methods. Results are expressed as a percentage of radioligand specifically bound in the absence of drugs and are means of three separate experiments performed in triplicate.

From these data, it was demonstrated that the binding sites of [^{3}H]bunazosin on rat white fat cell membranes have the characteristics of α_1 adrenergic receptors. These experiments demonstrate that the sites specifically labeled by [^{3}H]bunazosin are the physiological α_1-adrenoceptors and also that the ligand is reliable for the accurate identification of these receptors on rat white fat cell membranes.

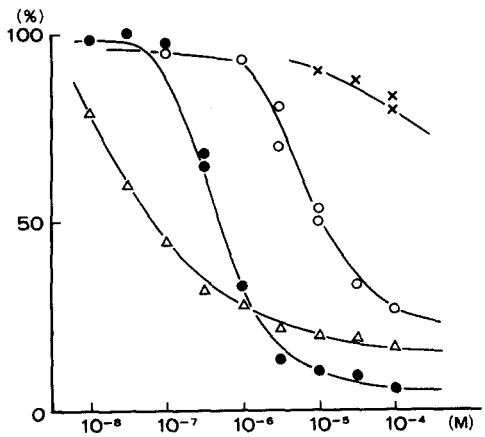


Fig. 4. Inhibition of [^{3}H]bunazosin binding using various agonists. Rat epididymal fat cell membranes (50–100 μg/ml) were incubated for 20 min at 25°C with [^{3}H]bunazosin (1 nM) and the indicated concentrations of l-norepinephrine (△), l-epinephrine (●), d-epinephrine (○), and isoproterenol (×). Binding assays were performed as described in Materials and Methods. Results are expressed as a percentage of radioligand specifically bound in the absence of drugs and are means of three separate experiments performed in triplicate.

[^{3}H]Bunazosin binding studies using aged obese rats

Using the same method as that for young rats, studies of [^{3}H]bunazosin binding to epididymal fat cell membranes from aged obese rats were performed. As shown in **Table 1**, the dissociation constant (K_d) was 0.63 ± 0.18 nM, which was not significantly different from that in young rats, the B_{max} was 24.0 ± 3.2 fmol/mg protein, which was significantly less than that in young rats ($P < 0.01$) (n = 6) (**Fig. 5**).

Studies of [^{3}H]rauwolscine and [^{3}H]CGP-12177 binding to epididymal fat cell membranes from young and aged rats

In order to detect the age-related changes in α_2- and β-adrenoceptors on epididymal fat cell membranes of SD rats, we used [^{3}H]rauwolscine and [^{3}H]CGP-12177 for α_2- and β-adrenoceptor binding studies, respectively.

In the studies with [^{3}H]rauwolscine, Scatchard analysis indicated a homogeneous population of binding sites with an equilibrium dissociation constant (K_d) of 0.72 ± 0.10 nM and 0.83 ± 0.04 nM in young and aged rats, respectively. B_{max} in aged rats was remarkably higher than that in young rats as shown in Fig. 5 (86.2 ± 19.5 vs. 19.8 ± 4.9 fmol/mg protein, respectively, $P < 0.01$).

As also shown in Fig. 5, in the studies of [^{3}H]CGP-12177 binding to rat white fat cell membranes, specific binding was also saturable and of high affinity. The equilibrium dissociation constants (K_d) showed no difference between young and aged rats (0.70 ± 0.15 vs. 0.79 ± 0.50 nM), while B_{max} values were significantly higher in young rats than in aged rats (69.7 ± 9.7 vs. 45.4 ± 13.9 fmol/mg protein, $P < 0.05$).

DISCUSSION

In this study, we used a newly developed radioligand, [^{3}H]bunazosin, which has been suggested as a probe to

TABLE 1. Age-related changes of K_d and B_{max} in binding experiments using ^{3}H-labeled radioligands

Radioligand	n	K_d	B_{max}
		nM	*fmol/mg protein*
[^{3}H]Bunazosin			
Young rat	6	0.58 ± 0.03	37.0 ± 6.5
Aged rat	6	0.63 ± 0.18	24.0 ± 3.2
P		NS	<0.01
[^{3}H]Rauwolscine			
Young rat	4	0.72 ± 0.10	19.8 ± 4.9
Aged rat	4	0.83 ± 0.04	86.2 ± 19.5
P		NS	<0.01
[^{3}H]CGP-12177			
Young rat	5	0.70 ± 0.15	69.7 ± 9.7
Aged rat	5	0.79 ± 0.50	45.4 ± 13.9
P		NS	<0.05

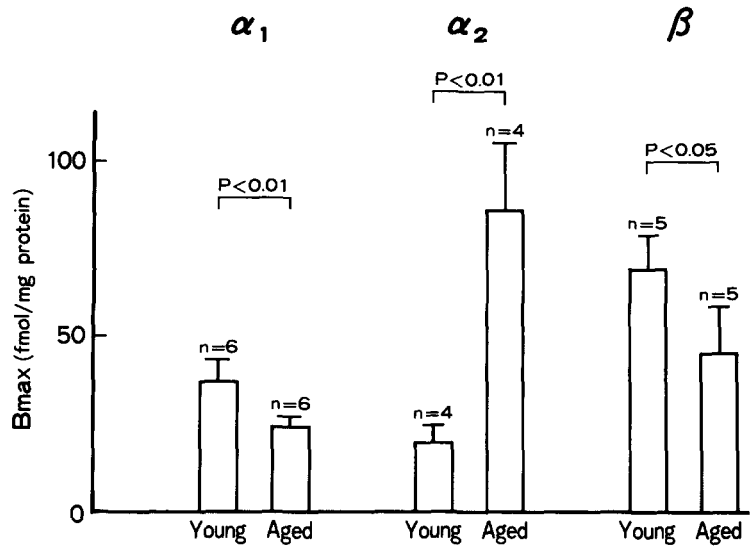


Fig. 5. The changes of adrenergic receptor number of rat white fat cell membranes with aging. The radioligands used were: α_1-adrenoceptor, [^{3}H]bunazosin; α_2-adrenoceptor, [^{3}H]rauwolscine; and β-adrenoceptor, [^{3}H]CGP-12177. Nonspecific bindings were defined with 10 μM unlabeled prazosin, yohimbine, and propranolol, respectively.

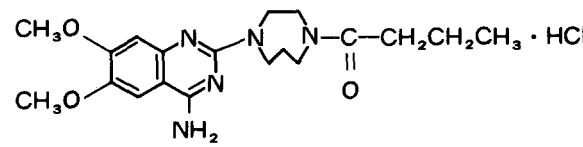
selectively identify α_1-adrenergic receptors (16), for investigating the α_1-adrenoceptors on rat white fat cell membranes. Phosphatidylinositol turnover, the entry or mobilization of calcium, and inhibition of glycogen syntheses (17) have been demonstrated by activation of α_1-adrenoceptors on membranes of rat adipocyte.

Brown adipocytes have been reported to possess α_1-adrenoceptors (18) and to show an α_1-component of norepinephrine-stimulated respiration (thermogenesis) (19, 20).

The existence of α_1-adrenoceptors on white fat cells of rats has also been confirmed by demonstrating the response to α_1-adrenergic receptor agonists (3), although the physiological role of this response was not clarified. The α_1-adrenoceptors on white fat cell membranes of rats, however, have not been detected by direct radioligand binding experiments. The reason for this might be the lack of a suitable radioligand to detect α_1-adrenoceptors on rat white fat cells. [^{3}H]Bunazosin, which facilitated the detection of α_1-adrenoceptors on white fat cell membranes in this study, is more hydrophilic than [^{3}H]prazosin, which we confirmed to be unsuitable for α_1-adrenoceptor binding to these membranes. Bunazosin is a quinazoline derivative with α_1-adrenoceptor blocking activity which was recently developed in Japan. Its chemical name is 4-amino-2-(4-butyrylhexahydro-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-quinazoline hydrochloride (**Fig. 6**). The chemical structure of prazosin is also shown in Fig. 6. As for the [^{3}H]bunazosin binding site, it has already been demonstrated to attach to α_1-adrenoceptor selectively (16). The binding of [^{3}H]bunazosin to rat white fat cell membranes was rapid, saturable, and reversible, and showed stereoanalog-specificity, by which the binding sites were found to possess the characteristics of α_1-adrenoceptors.

Since the classification of β-adrenergic receptors into β_1 and β_2 subtypes, additional β-adrenergic receptors have been implicated. Arch et al. (21) described new β-adrenoreceptor agonists (BRL26830A, BRL33725A, and BRL35135A) which selectively stimulate lipolysis in adipocytes. These agonists react mainly with the new so-called β_3-adrenoceptors which do not belong to the β_1- and β_2-subtypes. CGP12177 was used as a β-adrenoceptor agonist with no subtype selectivity. Thus, we could not detect the number of subtypes of β-adrenoceptors that had changed.

bunazosin



prazosin

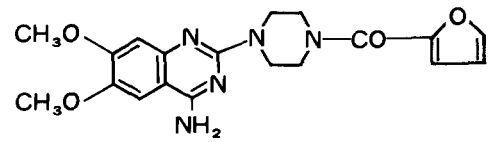


Fig. 6. Structures of bunazosin hydrochloride and prazosin. Bunazosin hydrochloride: 4-amino-2-(4-butyrylhexahydro-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-quinazoline hydrochloride. Prazosin: 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine.

As to age-related changes in α_1-adrenoceptors on rat epididymal fat cell membranes, the receptor number was shown to decrease significantly in aged rats in this study.

There are two opinions regarding age-related changes in β-adrenoceptors on rat fat cells. One is that the number of β-receptors markedly declines with senescence in rats (4), and the other is that β-adrenoceptor number remains the same in each rat age group (5). According to our present data, β-adrenoceptors were decreased in aged rats compared with those in young rats. On the other hand, the α_2-adrenoceptor binding sites on epididymal fat cell membranes in aged rats were increased approximately 4-fold relative to the number in young rats. The fat-cell volume in aged rats was about 2.3-fold that in young rats. Although the role of the α_1-adrenoceptor in lipolytic action has not been clarified, in general, stimulation of β-adrenoceptors on the fat cell enhances lipolytic action and stimulation of α_2-adrenoceptors on the fat cell suppresses its lipolytic action (1). Thus this volume change of the fat cells may be the result of changes in α_2- and β-adrenoceptor number. Judging from the degree of change in receptor number, α_2-adrenoceptors may be more dominant than β-adrenoceptors in the regulation of fat cell lipolysis, i.e., a strengthening of antilipolytic function mediated via α_2-adrenoceptors and a weakening of lipolytic function mediated via β-adrenoceptors so that fat cells tend to become engorged with lipid.

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