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CHARACTERIZATION OF THE β-ADRENERGIC TRANSDUCTION SYSTEM IN SPLEEN MONONUCLEAR LEUKOCYTE MEMBRANES OF YOUNG AND SENESCENT RATS

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Abstract—The effect of aging on some of the properties of the β-adrenergic transduction system was determined in a membrane fraction of spleen mononuclear leukocytes from young (2–3 months) and old (24–25 months) rats. Receptor density was unchanged and the percentage of receptors in the high affinity configuration for isoproterenol was reduced with increasing age. Adenylate cyclase activity, either unstimulated or stimulated with forskolin, GTP or isoproterenol was not affected by aging. This suggests the presence of compensatory mechanisms in the β-adrenergic signal transduction system of rat spleen mononuclear cells in order to ensure equal cAMP production for equal agonist stimulation.

It is generally accepted that aging is accompanied by a gradual decrease of the physiological and biochemical response to stimuli [1–3], although this is not always the case. Klein *et al.* [4] and Duckles *et al.* [5] e.g. demonstrated respectively for human vascular and non-vascular tissue and for vascular tissue of the rat, that the β-receptor mediated contractile response upon agonist stimulation was unchanged with age. Recently, we showed [6, 7] that in rat lung and kidney, the biochemical response, i.e. the adenylate cyclase activity upon agonist stimulation of the β-adrenoceptor was unaltered, while the β-adrenoceptor density increased and the percentage receptors in high affinity configuration decreased with age. We suggested that the latter changes are compensatory, in order to yield equal cAMP generation for equal stimuli in young and senescent rats. The first aim of this study was to investigate this hypothesis in rat spleen mononuclear leukocytes (MNL).

The second question addressed in this study is whether the age-related changes in the properties of the β-adrenergic system, that we observed in lungs and kidneys [6, 7], are reflected by similar changes in MNL. This is of particular interest since, in humans [8, 9] and in rats [10], peripheral blood lymphocytes are frequently used as a model for less accessible tissues. Additionally, we paid special attention to the link between age-related changes in properties of the β-receptor and changes in the ratio between the β_1- and β_2-subtypes. Indeed, we [7] and others [11] showed that in rat kidney aging can be accompanied by a change in subtype distribution, while in rat lung, this is not the case [6].

We investigated β-adrenoceptor density, ligand affinity, receptor-G-protein coupling, β-receptor subtype distribution and adenylate cyclase activity in basal and stimulated conditions. From the parameters measured, only the percentage of binding sites in high affinity configuration for isoproterenol was decreased in the old rats, suggesting that, as for lung and kidney, compensatory mechanisms exist. The results with mononuclear leukocytes however, only partly reflect what we found for lungs [6] and kidneys [7] of the rat, showing that findings in mononuclear cells can not automatically be extrapolated to less accessible tissues.

MATERIALS AND METHODS

Materials. [^{125}Iodo]cyanopindolol ([^{125}I]CYP, sp. act. 1972 Ci/mmol) and [8-^{3}H]adenosine-3′,5′-cyclic monophosphate ([^{3}H]cAMP, sp. act. 23 Ci/mmol) were obtained from Amersham (Amersham, U.K.). Adenosine-5′-trisphosphate (ATP), guanosine-5′-trisphosphate (GTP), creatine phosphokinase (E.C. 2.7.3.2) and creatine phosphate were obtained from Boehringer (Mannheim, F.R.G.). Adenosine-3′,5′-cyclic monophosphate (cAMP) was from Janssen Chimica (Beerse, Belgium). (−)-Isoproterenol hydrochloride, (−)-propranolol hydrochloride, forskolin and bovine serum albumin (BSA) were purchased from the Sigma Chemical Co. (Poole, U.K.). ICI 118,551 (erythro-*dl*-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) was a generous gift of ICI Pharma (Destelbergen, Belgium). Lymphoprep® (density 1.077 g/mL) and sodium metrizoate (density 1.200 g/mL) were from Nyegaard (Oslo, Sweden). RPMI-1640 medium was obtained from Gibco Europe (Ghent, Belgium). All other chemicals were of the highest purity available and obtained from local suppliers.

Animals. Male specific pathogen free (SPF) rats of 2 to 3 months and 24 to 25 months of age were obtained from the Proefdierencentrum (Centre for experimental animals) of the Katholieke Universiteit of Leuven (Belgium). They were housed individually

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and had free access to food and acidified water; they were fasted overnight before experiments.

Collection of spleens and mononuclear leukocyte membrane preparations. Animals were killed by decapitation. Spleens were quickly removed and placed at room temperature in RPMI-1640 adjusted to 310 mOsm with 2 M NaCl. The spleens were squeezed with the flat ended plunger of a syringe and RPMI-1640 was added to a final volume of 40 mL per spleen. Rat spleen mononuclear leukocytes were isolated essentially according to Böyum [12], with minor modifications. The obtained suspension was, after removal of connective tissue, layered in portions of 20 mL on 15 mL Lymphoprep adjusted to a density of 1.080 g/mL with sodium metrizoate. After centrifugation (40 min at 600 *g*) the interphase was collected, diluted to 50 mL with 0.9% NaCl, and centrifuged for 30 min at 600 *g*. The pellet was again resuspended in 40 mL 0.9% NaCl solution and centrifuged for 20 min at 500 *g*. The obtained leukocyte pellet was resuspended in 3 mL 0.9% NaCl and cells were counted in a Bürker chamber. At least 1×10^8 mononuclear cells with a viability of at least 96% (as determined with the trypan blue exclusion test) were recovered per spleen.

The cells were lysed in 5 mL ice-cold 5 mM Tris–HCl, 0.2 mM EGTA, pH 7.40 for 5 min. All further procedures were performed at 4°. After lysis, the remaining fragments were disrupted with a Virtis homogenizer (70% of maximal speed, 3 × 15 sec) and diluted with 5 mL 0.5 M sucrose, 100 mM Tris–HCl, 4 mM EGTA, pH 7.40. The membranes were collected by centrifugation at 60,000 *g* for 40 min. The pellet was finally resuspended in 50 mM Tris–HCl, 20 mM $MgCl_2$, 2 mM EGTA, pH 7.40 (assay buffer). Protein determinations were done with the dye binding method according to Macart and Gerbaut [13] using BSA as a standard.

Ligand binding experiments. The total number of β-adrenergic receptors (B_{max} in fmol/mg membrane protein) and their equilibrium dissociation constant (K_d in pM) for [^{125}I]CYP were determined in spleen MNL from eight young and seven old rats using saturation binding experiments as described before [14]. Membrane proteins (9–12 μg in assay buffer) were incubated with eight concentrations of [^{125}I]CYP (from 1 to 350 pM) in a total volume of 250 μL for 60 min at 37°. Non-specific binding was defined as the radioactivity not displaced by 10 μM of (−)-propranolol.

Competition binding experiments were performed for the determination of the β-receptor subtype distribution in five young and six old animals and for the detection of different affinity states of the receptors for (−)-isoproterenol in 18 young and 16 old animals. MNL membranes (9–12 μg protein in assay buffer) were incubated with a single [^{125}I]CYP concentration (varying between 90 and 160 pM) and 12 concentrations of either the β_2-selective antagonist ICI 118,551 (0.01 nM–0.1 mM) or the non-selective agonist (−)-isoproterenol (0.1 nM–1 mM) at 33° for 60 min. In the experiments with (−)-isoproterenol the incubation mixtures were supplemented with 0.01% ascorbic acid.

All incubations were done in duplicate in microtiter plates and were stopped by rapid vacuum filtration over Gelman A/E glass fiber filter sheets (25.4 × 3.5 cm). The filters were rinsed 10 times with 300 μL 0.9% NaCl solution using the semi-automatic cell harvester filtration method [14]. The filters were counted in 4 mL Ultima Gold (Packard) with 65–75% counting efficiency in a Liquid Scintillation Spectrometer (type 4530) [15].

Stimulation of adenylate cyclase and cAMP determination. All methods for stimulation of adenylate cyclase and determination of formed cAMP were described before [6]. Membrane suspensions (100 μL containing 15–20 μg of protein) were added to the incubation mixtures, which contained either 33 μM GTP, 25 μM forskolin, 10 mM NaF or 33 μM (−)-isoproterenol together with GTP. In a separate series of experiments, dose–response curves for isoproterenol (concentration range from 5×10^{-10} to 1×10^{-4} M) in the presence of a fixed concentration of GTP (10^{-4} M) and for GTP (concentration range from 1×10^{-9} to 1×10^{-4} M) were investigated. After incubation (10 min at 30°), the samples were diluted with 750 μL 10 mM Tris–HCl buffer, pH 7.5, containing 2 mM EDTA, heated (15 min at 100°), cooled to 4° and centrifuged (5000 *g* for 10 min). The cAMP content in the supernatant was determined by a protein binding assay [16] as described before [6]. The between assay coefficient of variation, determined with a home-made cAMP standard of 7.7 ± 0.7 pmol/tube, was 9.3% (N = 11).

Calculations. Receptor density and ligand affinity were calculated from saturation binding experiments after transformation to Scatchard plots. The correlation coefficient for the linear regression was usually greater than 0.9; only data from curves with $r > 0.85$ were used for further calculations. Competition binding curves were analysed with "GraphPAD" [17]; K_i values were calculated according to Cheng and Prusoff [18]. Results are presented as means ± SE. The two tailed Student's *t*-test was used for the estimation of significant differences between the two age groups, the two tailed paired *t*-test for comparisons within one age group.

RESULTS

Representative examples of the saturation binding experiments with spleen mononuclear leukocytes from young and old rats are shown in Figs 1 and 2 and the results are summarized in Table 1. In all cases, linear Scatchard plots are obtained. At the highest ligand concentration, non-specific binding was 23.5 ± 4.1 and 17.8 ± 2.4% of total binding in young and old rats, respectively ($P > 0.05$). Neither receptor density nor equilibrium dissociation constant are different between both age groups.

The results of the competition binding experiments with (−)-isoproterenol are shown in Fig. 3. In both age groups, all curves are shallow and the presence of a high and low affinity configuration could be calculated. In young animals, 35.9 ± 3.3% and in old animals 25.3 ± 2.7% of the receptors are in high affinity configuration ($P < 0.05$).

The results of the competition binding experiments with ICI 118,551 are shown in Fig. 4. No difference is found between both age groups: 63.0 ± 2.7% (N = 5) and 64.5 ± 2.6% (N = 6) of the total receptor

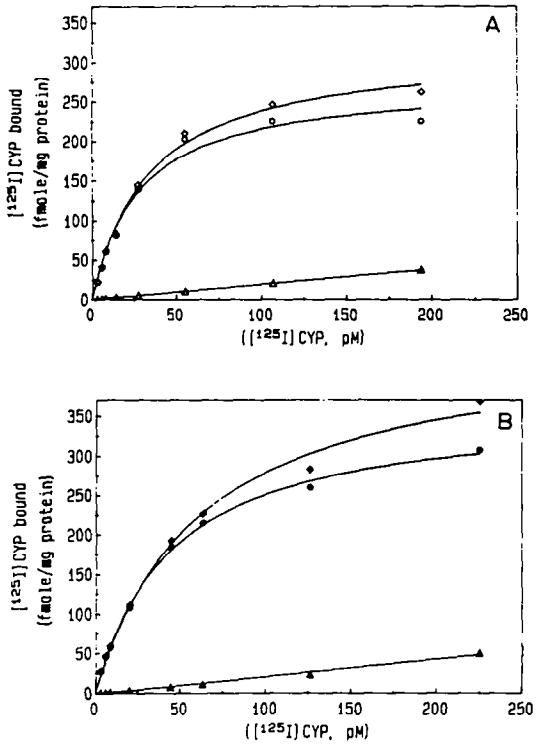

Fig. 1. Saturation binding curves with rat spleen mononuclear leukocyte membrane preparations are shown. A representative example for (A) a young rat (2 months, empty symbols) and (B) an old rat (24 months, filled symbols) is shown. Diamonds, total binding; triangles, non-specific binding; circles, specific binding.

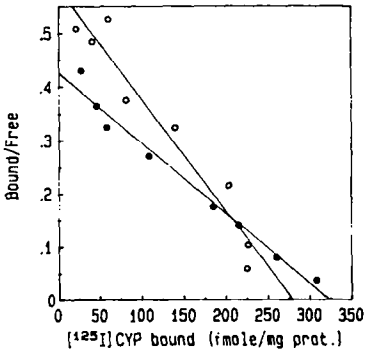

Fig. 2. Scatchard plot of the results shown in Fig. 1, Empty symbols: young rat; B_{max}, 279.9 fmol/mg protein; K_d, 22.8 pM; $r = 0.97$. Filled symbols: old rat; B_{max}, 323.4 fmol/mg protein; K_d, 28.3 pM; $r = 0.99$.

population bound the ICI compound with high affinity in young and old animals, respectively.

The results of the determination of rat spleen mononuclear leukocyte adenylate cyclase activity are summarized in Table 2. All stimulatory conditions (GTP, NaF, forskolin or isoproterenol in the presence of GTP), significantly increase the activity of adenylate cyclase above basal activities for both age groups ($P < 0.01$). The net values obtained with GTP, NaF, forskolin (value in stimulatory condition minus value for the basal activity) or the net stimulation by isoproterenol (value obtained in the presence of GTP minus value with GTP alone) are not different between both age groups.

In Fig. 5, the concentration–response curves for the stimulatory effect of GTP (N = 4, Fig. 5A) and of isoproterenol in the presence of GTP (N = 4, Fig. 5B) on the activity of adenylate cyclase are shown. For both stimulatory conditions, the curves obtained using young and old MNL-membranes are almost superimposable; no difference between both age groups is found. The ED_{50}-values for the concentration–response curves with GTP are 1.18×10^{-6} M and 1.00×10^{-6} M, respectively, for MNL membranes obtained from young and old rats, and 4.45×10^{-6} M and 4.02×10^{-6} M for the curves with isoproterenol in the presence of GTP.

DISCUSSION

In this paper, we describe the properties of the β-adrenergic transduction system in a membrane preparation from spleen mononuclear leukocytes obtained from young and old rats.

The results show that there are few differences between both age groups. From the ligand binding experiments, we can conclude that the percentage of receptors in high affinity configuration for isoproterenol is significantly lower in old animals, while receptor density is unaltered. Similarly, the activities of adenylate cyclase upon stimulation with forskolin (active at the adenylate cyclase), with GTP or NaF (both active at the GTP-protein) or with isoproterenol in the presence of GTP (requiring the adequate functioning of the entire transduction system) are unchanged with age.

The data in the literature on the properties of the β-adrenergic system in rat MNL and on age-related changes in these properties, are rather limited. In our preparation the receptor density is approximately two to three-fold higher than what is found for peripheral MNL of the rat [10]. Whether this is due to a difference in tissue, in preparation of the tissue or in ligand binding methodology remains to be established. Abrass *et al.* [19] found that the density of β-receptors in rat spleen MNL was slightly lower in old animals, although statistical significance was not reached. De Blasi *et al.* [20] found no difference at all in receptor density in peripheral MNL between both age groups. Our results are similar in that there is only a tendency for an increased receptor density in preparations of older animals.

As far as adenylate cyclase is concerned, De Blasi *et al.* [20] determined isoproterenol-stimulated cAMP synthesis in peripheral leukocytes from young animals only. In our experiments, including the dose–response experiments, there is no difference between the two age groups. It has to be concluded that the biochemical function of the β-adrenergic transduction system in spleen MNL, in terms of synthesis of cyclic AMP upon agonist stimulation, is not affected by the aging process.

In our previous papers on rat lung and kidney [6, 7], we suggested that an increase in receptor density compensates for the decrease in percentage

Table 1. Results of binding experiments of [^{125}I]CYP on β-adrenoceptors of spleen mononuclear leukocyte membranes from young (2–3 months) and old (24–25 months) rats

	Young rats (N = 8)	Old rats (N = 7)
Saturation binding experiments		
B_{max} (fmol/mg protein)	267.1 ± 20.5	304.4 ± 49.9
K_d (pM)	20.5 ± 2.7	20.3 ± 2.2
Competition binding experiments with		
(−)-isoproterenol	(N = 18)	(N = 16)
High affinity binding (%)	35.9 ± 3.3	25.3 ± 2.7*
K_i High affinity (nM)	6.3 ± 1.6	5.8 ± 1.6
Low affinity binding (%)	64.1 ± 3.3	74.7 ± 2.7*
K_i Low affinity (nM)	375.8 ± 49.1	279.5 ± 42.2

B_{max}, number of binding sites.
K_d, equilibrium dissociation constant.
K_i, equilibrium inhibition constant.
* $P < 0.05$.

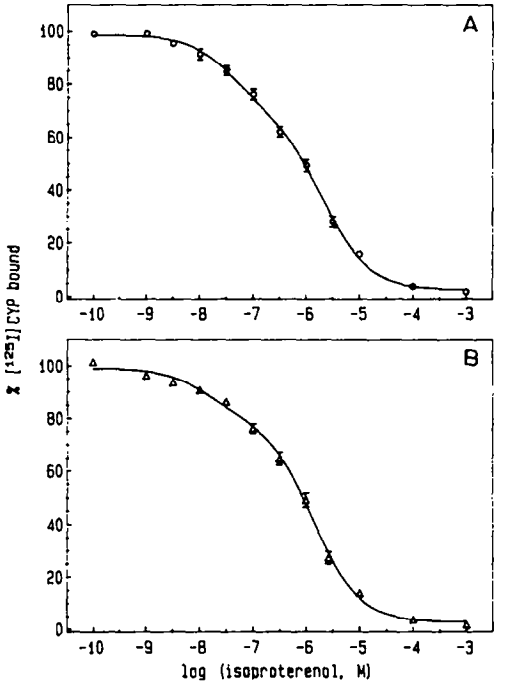

Fig. 3. Competition binding curves with (−)-isoproterenol as competitor and [^{125}I]CYP as ligand (means ± SE). (A) Young rats (2–3 months), N = 18; (B) old rats (24–25 months), N = 16.

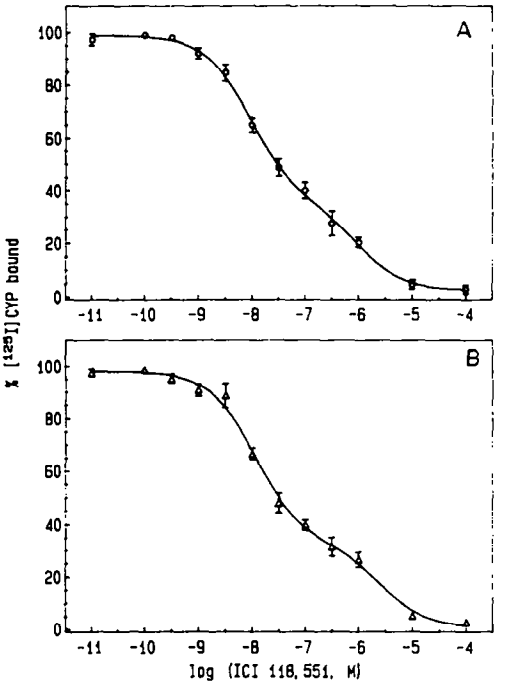

Fig. 4. Competition curves with ICI 118,551 as competitor and [^{125}I]CYP as ligand (means ± SE). (A) Young rats (2–3 months), N = 5; (B) old rats (24–25 months), N = 6.

receptors in high affinity configuration for agonists, in order to ensure equal cAMP synthesis for equal agonist stimulation. For rat spleen MNL however, the decrease of the percentage of receptors in high affinity configuration for isoproterenol is not accompanied by an increase in receptor density upon aging so that another compensatory mechanism seems involved in order to yield equal agonist-stimulated cAMP production. These differences between rat spleen MNL on the one hand and rat lung and kidney on the other indicate that findings in MNL cannot be automatically extrapolated to other tissues.

As far as β-receptor subtypes are concerned, we found that in rat spleen mononuclear leukocytes, using the highly specific β_2-antagonist ICI 118,551 [21], approximately 35 to 37% of the receptor population has a low affinity for the antagonist, hence, are probably of the β_1-subtype. There is no influence

Table 2. Net adenylate cyclase activity (pmol/mg protein min) of rat spleen mononuclear leukocyte membranes from young (2–3 months) and old (24–25 months) rats

	Young rats (N = 15)	Old rats (N = 13)
Stimulatory condition		
None (basal value)	36.4 ± 2.6	41.9 ± 4.2
GTP*	25.3 ± 2.7	32.8 ± 5.3
GTP + isoproterenol†	24.2 ± 2.3	26.6 ± 3.8
NaF*	45.7 ± 3.3	51.3 ± 5.5
Forskolin*	129.4 ± 7.6	153.7 ± 14.9

* Activities above basal value.
† Activities above values of stimulation with GTP.

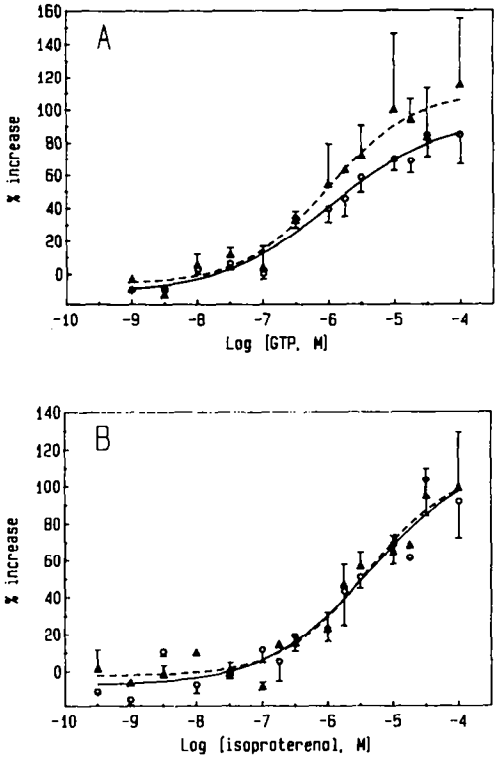

Fig. 5. Concentration–response curves of the effect of (A) GTP and (B) isoproterenol in the presence of GTP (1×10^{-4} M) on the activity of adenylate cyclase. Adenylate cyclase activity is expressed as percentage increase above the value obtained in the absence of GTP (Fig. 5A) or of isoproterenol (Fig. 5B). Open circles, solid lines: young rats (2–3 months), N = 4. Filled triangles, dashed lines: old rats (24–25 months), N = 4.

of age on the β_1/β_2-ratio. Compared to our previous results, the β_1/β_2-ratio in rat spleen MNL is very similar to what is found in rat lung (30% β_1) and different from the situation in rat kidney (70% β_1). Additionally, upon aging, the ratio between the two subtypes in kidney is changed in favour of the β_2-subtype [7], while in lung [6] and in MNL (this study), the ratio is unchanged.

Brodde *et al.* [22] concluded recently that in humans, the use of β_2-receptors in lymphocytes as a model for predicting the properties of heart β-receptors and the changes hereof is of limited use, since the cardiac β-receptors are predominantly of the β_1-subtype. Our results suggest that, even when the β_1/β_1-ratio is the same (e.g. as in rat MNL and in lung tissue), changes in properties of the β-adrenergic transduction system are not comparable.

Taken together, we have to conclude from these results that rat spleen MNL as a model for the study of age-related changes in the properties of the β-adrenergic transduction system for other tissues should be used with care.

Our results further illustrate the clearcut species difference in the β_1/β_2-ratio in MNL. Indeed, in human MNL only β_2-receptors are found [23, 24], in rabbits, 50% of the β-receptors are shown to be of the β_1-subtype [25], while we find that in rat MNL 35% of the receptors are of the β_1-subtype.

We conclude that age affects the properties of the β-adrenergic transduction mechanism in spleen MNL to a very small extent; the only difference is a lowered percentage of the receptors in high affinity configuration for agonists. The observation that at the same time, the cAMP production upon isoproterenol stimulation is similar in both age groups, suggests again the presence of homeostatic mechanisms.

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