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THE EFFECT OF AGE AND CHOLESTEROL ON THE RAT LUNG BETA-ADRENERGIC SYSTEM

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To assess the influence of membrane lipid composition on beta-adrenergic receptor number and adenylate cyclase activity in aging, we investigated the effect of cholesteryl hemisuccinate on these parameters in lung membranes of 3-, 12-, and 24-month-old CDF (F-344) rats. When cholesteryl hemisuccinate (0.5 mg/ml) was incubated with lung membranes, beta-adrenergic receptor density was increased by 70%. This effect was the same for each age group studied and indicated that the density of both basal and CHS-sensitive receptors is unaltered in rat lung with age. Forskolin, NaF, p[NH]ppG, and isoproteronol-stimulated adenylate cyclase activity is 30% lower in lung membranes from aged rats. Since enzyme activity is affected by the lipid environment and membrane composition often changes with age, we assessed adenylate cyclase activity following cholesteryl hemisuccinate incorporation. There was up to a 75% decrease in adenylate cyclase activity following cholesteryl hemisuccinate incorporation in lung mebranes in each of the three age groups. In untreated membranes, there was no significant difference in cholesterol or lipid phosphate content with age. These data suggest that cholesterol content does not account for alterations in senescent rat lung adenylate cyclase activity.

Introduction

Loss of beta-adrenergic responsiveness is known to occur in senescence. The inotropic and chronotropic responses of the heart to catecholamines are decreased in older rats [1,2], as is the chronotropic response in older dogs [3] and elderly humans [4]. Two sites along the beta-adrenergic pathway have been identified to functionally decrease with age. While there is no difference in beta-adrenergic receptor density [1,2,5], receptor agonist affinity and adenylate cyclase activity decrease in rat heart membranes with age [5–8]. Similarly, in rat lung, we have shown a decrease in receptor agonist affinity and diminished adenylate cyclase activity with age while receptor number is unaltered [9].

There are marked differences between young and old animals in the cholesterol to phospholipid ratio in the membranes of various tissues [10,11]. Some of the properties of membrane-bound enzymes and receptors are altered by changes in their lipid environment, therefore it is possible that some of the differences in the beta-adrenergic pathway with age are secondary to alterations in membrane composition. Membranes from senescent mouse brain have 50% more serotonin binding sites than membranes from younger animals. The number of serotonin binding sites can be markedly increased by incorporation of cholesteryl hemisuccinate or stearic acid into membranes [12]. When mouse brain membranes are treated with

Abbreviation: p[NH]ppG, guanosine 5'-imidotriphosphate.

cholesteryl hemisuccinate, serotonin binding increases 7-fold in young and only 3-fold in old mice; thus the total membrane binding capacity of young is about double that of old mice [13]. These data suggest that membrane lipid composition may be related to some of the alterations of aging.

Beta-adrenergic receptor number can also be altered by changes in membrane composition. A 15-fold increase in beta-adrenergic receptor number was reported in mouse brain membranes after the in vitro addition of cholesteryl hemisuccinate [13]. Several other reports have analyzed beta-adrenergic receptor density after changes in membrane lipid composition and viscosity. Following fluidization, Chang liver cell membranes exhibit decreased beta-adrenergic receptor density [14]; rat reticulocyte mebranes fluidized following phospholipid methylation demonstrate increased receptor density [15]; and turkey erythrocyte membranes exposed to a variety of agents exhibit no change in receptor density [16]. The present study examines beta-adrenergic receptors following incorporation of cholesteryl hemisuccinate into lung membranes from young and old rats.

The lateral movement of components of the adenylate cyclase system is important for the activity of the enzyme, and a decrease in the fluidity of the membrane can result in diminished enzyme activity [16]. Since membrane composition often changes with age, the reduced adenylate cyclase activity in the membranes of older animals may be a consequence of changes in their lipid content. Therefore, we assessed lung membrane adenylate cyclase activity in 3-, 12-, and 24-month-old rats following in vitro incubation with cholesteryl hemisuccinate. In addition, the cholesterol and phosphate content of the membranes was measured.

Methods

Animals. Female CDF (F-344) rats of 3, 12, and 24 months of age were obtained from Charles River Breeding Laboratory (North Wilmington, MA, U.S.A.) under contract with the National Institute on Aging.

Membrane preparation. Rats were killed by cervical dislocation under pentobarbitol anesthesia. The circulatory system was perfused with 10 ml cold saline and the lung excised and minced in 15 ml of cold 0.24 M sucrose, 1 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.4). Preparations were exposed to two 15-s bursts of a Tekmar Tissuemizer and homogenized with 10 strokes of a motor-driven Teflon-tipped pestle at moderate speed. The homogenate was filtered through four layers of cheese cloth and centrifuged at $48\,000 \times g$ for 15 min. The pellet was suspended in 10 ml of 50 mM Tris-HCl (pH 7.4) and quick frozen in liquid nitrogen.

Cholesteryl hemisuccinate incorporation into lung membranes. Lung homogenate (1 mg/ml) was incubated for 2 h at room temperature in 45 mM Tris-HCl (pH 7.4), 9.5% ethanol, and cholesteryl hemisuccinate (0–0.5 mg/ml). If adenylate cyclase activity was to be measured, 10 mM NaF was included in the incubation to stabilize the enzyme. After incubation, the solution was centrifuged at $48\,000 \times g$ for 15 minutes and the pellet suspended in 50 mM Hepes (pH 7.4), 0.08 mM ascorbic acid, and 18 mM $MgCl_2$.

Beta-adrenergic receptor assay. Beta-adrenergic receptors were analyzed as previously reported [19]. Briefly, di[^{3}H]hydroalprenolol (spec. act. 49.1 Ci/mmol; New England Nuclear Corp., Boston, MA) binding was assayed by incubating 100 μg of lung membrane for 15 min at 37°C with eight different concentrations of di[^{3}H]hydroalprenolol ranging from 0.5 to 5 nM in a total volume of 250 μl. A duplicate set of tubes containing 10^{-6} M propranolol was also incubated to determine nonspecific binding. Receptor density and affinity were analyzed by the method of Scatchard.

Adenylate cyclase assay. Approx. 75 μg of membrane protein were assayed for adenylate cyclase activity as described earlier [6]. In some tubes either 10 mM NaF, 10^{-4} M p[NH]ppG, 33.3 μM forskolin, or 33.3 μM forskolin and 10^{-5} M GTP and 10^{-3} M isoproterenol were present as enzyme stimulants. Protein was determined by a modification of the biuret method [6].

Lipid determinations. Lung membrane pellets were extracted with chloroform/methanol (2 : 1, v/v) according to the method of Frolich et al. [17]. The extract was washed with an equal volume of water. A portion of the organic phase was dried, ashed and analyzed for phosphate by the method of Chen et al. [18]. A second portion of the extract

was analyzed for cholesterol by the enzymatic assay of Allain et al. (Sigma Chemical Co. Kit No. 350-A, St. Louis, MO).

Membrane fluidity. Membrane fluidity was determined by electron paramagnetic resonance in the laboratory of James H. Armbrecht [19].

Results

There is a dose dependent increase in beta-adrenergic receptor density when rat lung membranes are preincubated with cholesteryl hemisuccinate (Fig. 1). Preincubation with a cholesteryl hemisuccinate concentration of 0.5 mg/ml membrane was associated with a 70% increase in the number of beta-adrenergic receptors compared to untreated lung membranes (Fig.1). The increase in receptor number was not associated with any change in antagonist affinity. To assess the effect of age and cholesteryl hemisuccinate incorporation on beta-adrenergic receptor characteristics, lung membranes from 3-, 12-, and 24-month-old rats were preincubated with a maximal concentration of cholesteryl hemisuccinate. Scatchard analysis of di[^{3}H]hydroalprenolol binding from a single experiment is shown in Fig. 2. There is no difference in beta-adrenergic receptor density with age in untreated lung membranes. Cholesteryl hemisuccinate treatment increases receptor density to the same extent in all age groups (Fig. 2, Table I). Furthermore, there is no difference in beta-adrenergic receptor antagonist affinity with either age or preincubation with cholesteryl hemisuccinate (Fig. 2, Table I).

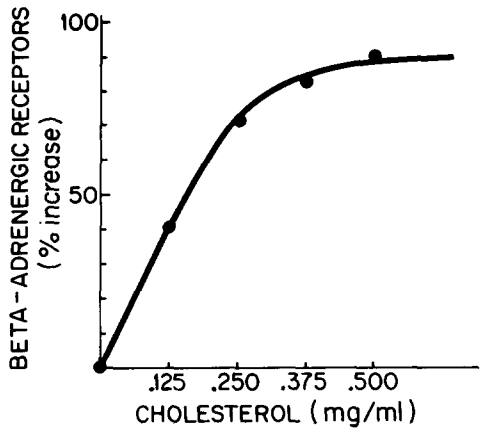

Fig. 1. Dose-dependent increase in receptor density following incorporation of cholesteryl hemisuccinate. Lung membranes were incubated with increasing concentrations of cholesterol (0–0.5 mg/mg protein) for 2 h at room temperature, followed by one wash. From 0–0.26 mg cholesterol per mg protein were incorporated. The order parameter, *S*, (a measure of membrane fluidity) increased from 0.596 in the absence to 0.665 in the presence of 0.5 mg/ml cholesterol, indicating an increase in membrane rigidity. Each point represents receptor number determined by eight-point Scatchard analysis.

Adenylate cyclase activity in untreated lung membranes of 3-, 12-, and 24-month-old rats shows a progressive age-related decline in basal, NaF, p[NH]ppG, and isoproterenol-stimulated activity (Table II). To assess the effects of membrane cholesterol on adenylate cyclase activity, cholesteryl hemisuccinate was incubated with lung membranes and the resulting enzyme activity measured. In contrast to changes in beta-adren-

TABLE I

THE EFFECT OF AGE AND CHOLESTERYL HEMISUCCINATE ON BETA-ADRENERGIC RECEPTOR DENSITY AND DIHYDROALPRENOLOL AFFINITY

Membranes were incubated with and without cholesteryl hemisuccinate (0.5 mg/ml) for 2 h at room temperature. Receptor density and antagonist affinity were determined from Scatchard plots. Data represents the mean ± S.E. of six animals.

Age	Control		Cholesterol	
	Density (fmol/mg protein)	K_d (M) ($\times 10^9$)	Density (fmol/mg protein)	K_d (M) ($\times 10^9$)
3 month	133 ± 14	0.83 ± 0.12	216 ± 12	0.85 ± 0.12
12 month	137 ± 10	0.69 ± 0.10	209 ± 12	0.63 ± 0.08
24 month	154 ± 24	0.75 ± 0.13	205 ± 20	0.60 ± 0.10

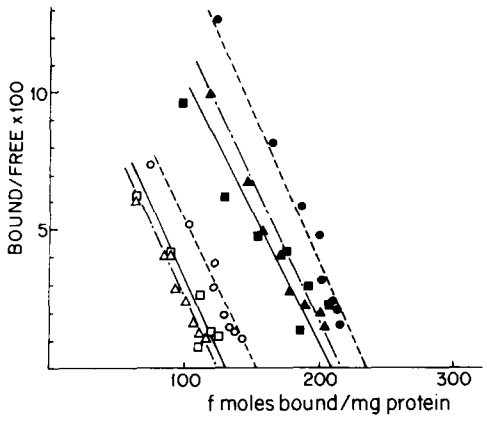

Fig. 2. Scatchard analysis of di[^{3}H]hydroalprenolol binding to lung membranes from 3-month (□, ■), 12-month (△, ▲), and 24-month (○, ●) old rats preincubated without (open symbols) or with (closed symbols) 0.5 mg cholesteryl hemisuccinate per ml. Lines were determined by regression analysis with correlation coefficients > 0.98. Receptor number is determined by abscissa intercept and K_d from the negative reciprocal of the slope of the line.

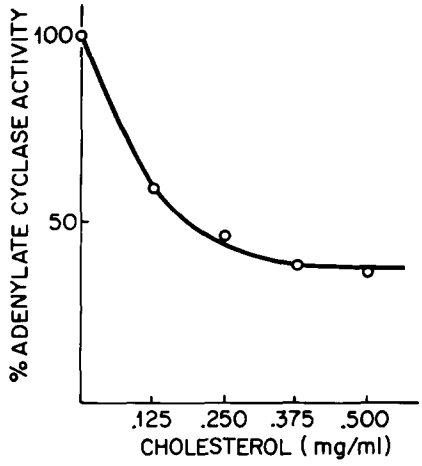

Fig. 3. Dose-dependent decrease in adenylate cyclase activity following incorporation of cholesteryl hemisuccinate. Lung membrane was incubated with increasing concentrations of cholesteryl hemisuccinate (0–0.5 mg/mg protein) for 2 h at room temperature in the presence of 10 mM NaF. After one wash, membranes were assayed for adenylate cyclase activity.

ergic receptor density, there is a dose-dependent decrease in NaF-stimulated adenylate cyclase activity following cholesteryl hemisuccinate incorporation (Fig. 3). At half maximal inhibition of enzyme activity, 0.23 μmoles cholesteryl hemisuccinate are incorporated per mg membrane protein; while at maximal inhibition, 0.66 μmoles cholesteryl hemisuccinate are incorporated per mg membrane protein. This represents five times the intrinsic membrane concentration and enzyme activity measured under these conditions should be independent of the membrane's initial cholesterol content. Preincubation with maximal concentrations of cholesteryl hemisuccinate decreases basal as well as isoproterenol, NaF, and p[NH]ppG stimulated adenylate cyclase activity up to 75% (Table II). The decrease was similar in all age groups, and the pattern of diminished enzyme activity with age was the same in both the untreated and cholesteryl hemisuccinate-treated membranes

TABLE II

THE EFFECT OF AGE AND CHOLESTERYL HEMISUCCINATE ON ADENYLATE CYCLASE ACTIVITY

Adenylate cyclase activity was assessed following a preincubation with or witout cholesteryl hemisuccinate (0.5 mg/ml) at room temperature for 2 h. The data represent the mean ± S.E. of six animals.

Stimulant	Adenylate cyclase activity (pmol cAMP/min/mg protein)					
	3 Month		12 Month		24 Month	
	Control	Cholesterol	Control	Cholesterol	Control	Cholesterol
None	41 ± 9	10 ± 4	32 ± 7	8 ± 4	30 ± 6	5 ± 2
NaF	113 ± 24	53 ± 14	87 ± 17	31 ± 9	70 ± 13	23 ± 5
p[NH]ppG	82 ± 18	29 ± 8	67 ± 14	21 ± 6	49 ± 10	13 ± 3
Forskolin	55 ± 13	14 ± 5	48 ± 12	9 ± 4	37 ± 9	7 ± 3
Isoproterenol	82 ± 21	27 ± 9	64 ± 15	15 ± 2	50 ± 7	15 ± 5

TABLE III

THE EFFECT OF AGE ON THE CHOLESTEROL AND LIPID PHOSPHATE CONTENT OF RAT LUNG MEMBRANES

Data represent the mean ± S.E. of six animals.

Age (month)	Cholesterol (μmol/mg protein)	Phosphate (μmol/mg protein)	Ratio
3	0.129 ± 0.015	0.628 ± 0.023	0.205
12	0.132 ± 0.008	0.620 ± 0.017	0.213
24	0.125 ± 0.018	0.561 ± 0.017	0.223

(Table II). In addition, the cholesterol and phosphate content of chloroform-methanol extracts of lung membranes from 3-, 12- and 24-month-old rats was measured and found to be unaltered with age (Table III).

Discussion

Decreased responsiveness to a variety of hormones including catecholamines is a well-documented phenomenon of aging [20]. To elucidate the mechanism of reduced catecholamine responsiveness, numerous components of the beta-adrenergic pathway have been investigated for decreased function with age. One component, beta-adrenergic receptor density, is unchanged with age in rat heart and lung, and human lymphocytes [1,2,5,9,21]. However, this methodology may not measure all available beta-adrenergic binding sites. For example, the addition of cholesterol to rat brain membranes increases measurable serotonin and beta-adrenergic binding sites [13]. This study demonstrated an age-related decrease in cholesterol-sensitive serotonin binding. In the present study we demonstrate that cholesteryl hemisuccinate enhances di[^{3}H]hydroalprenolol binding to lung membranes. However, our results, indicate that beta-adrenergic receptor density is the same in all age groups both before and after cholesteryl hemisuccinate treatment. While it is unclear what role these cholesteryl hemisuccinate-dependent receptors play in the biologic response to catecholamines, these data suggest that cryptic receptors exposed following incubation with cholesteryl hemisuccinate do not contribute to the diminished catecholamine responsiveness in lungs of aging rats.

While rat lung beta-adrenergic receptor density does not change with age, there is a 30% loss in adenylate cyclase activity as rats age from 3 to 24 months. It is possible that this decrease is a consequence of changes in membrane lipid composition with age. Adenylate cyclase activity is reduced following membrane cholesterol enrichment in rat kidney fibroblasts [22]; however, in human platelet membranes, NaF-stimulated enzyme activity is either not different [23] or decreased [24] with cholesterol incorporation. It is also possible that other important components of the membrane, such as the ratio of saturated to unsaturated fatty acids, could vary altering membrane fluidity and enzyme activity. In the present study, however, we found no changes in either membrane cholesterol or lipid phosphate content of lungs from 3-, 12-, and 24-month-old rats. This evidence suggests that cholesterol content is not related to changes in adenylate cyclase activity with age. Under the conditions of excess cholesteryl hemisuccinate, the measurement of adenylate cyclase activity is independent of the intrinsic membrane cholesterol content. When the membranes from young and old rats were treated with a maximal concentration of cholesteryl hemisuccinate, the adenylate cyclase activity remaining after cholesteryl hemisuccinate incorporation still demonstrated the same age-dependent decrease in adenylate cyclase activity. In addition, from the dose response curve, we have determined that it would require a 2-fold increase in membrane cholesterol to reduce enzyme activity by the 30% loss seen with age. Thus, these data do not support the hypothesis that the changes which occur in the adenylate cyclase system with age are secondary to changes in membrane cholesterol content.

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