Effects of Age on β Adrenergic Subtype Activation of Adenylyl Cyclase in Brown Adipose Tissue (44058)

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Abstract. **Thermogenesis in brown adipose tissue (BAT) is believed to be mediated mainly by β_3 adrenergic receptors. We previously demonstrated that the specific β_3 adrenergic agonist CGP-12177 increases whole body oxygen consumption and BAT GDP binding to a greater extent in young than in senescent rats. In contrast, the forskolin-induced increases were maintained with age, suggesting that early events in β_3 adrenergic signal transduction are impaired with age. To investigate whether β_1 or β_3 adrenergic function is decreased with age, we assessed β_1 and β_3 adrenergic receptor mRNA levels and the ability of β_1 and β_3 adrenergic receptors to activate adenylyl cyclase in BAT membranes from 4- and 24-month-old F-344 rats. Both β_1 and β_3 adrenergic receptor mRNA levels decreased by 50% with age. Adenylyl cyclase stimulated by the nonspecific agonist, isoproterenol, and by the specific β_3 agonist, BRL 37344, also declined by 50% with age, whereas glucagon stimulation decreased by more than 70%. The isoproterenol-stimulated adenylyl cyclase activation curves were resolved by two-site regression analysis to determine the contribution of β_1 and β_3 adrenergic receptors. The V_{max} for both β_1 and β_3 adrenergic receptors decreased by 50% with age. However, stimulation of adenylyl cyclase by NaF and forskolin was also diminished by the same amount as β adrenergic stimulation, suggesting that the activation with age may be limited by the amount of adenylyl cyclase catalytic unit rather than by receptor number. These data suggest both β_1 and β_3 adrenergic receptors and adenylyl cyclase catalytic units are deficient with age in rodent BAT.**

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In rodents, thermoregulation is impaired with age and varies according to the severity of the cold stress, as well as the age, strain, and gender of the animal (1–3). In the rat, nonshivering thermogenesis in brown adipose tissue (BAT) is an important contributor of the heat necessary to maintain body temperature. BAT thermogenesis is mediated by catecholamine activation of adenylyl cyclase through sympathetically innervated β adrenergic receptors (4). With age, sympathetically activated thermogenesis in BAT is attenuated and may account for the loss of thermoregulation in senescence. For example, we and others have reported that norepinephrine-stimulated O_2 consumption, one measure of thermogenesis, is decreased in senescent rats (1, 3). Moreover, we reported that in senescent rats β adrenergic signal transduction in BAT is impaired (5). Recent evidence suggests that the β adrenergic receptor (AR) in BAT is pharmacologically distinct from either the β_1 or the β_2 adrenergic subtypes and predominantly consists of the atypical β_3 adrenergic subtype (6, 7). Although the specific subtype quantification of the β_3 adrenergic receptor has not been determined in young compared

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with senescent rats, the total number of β adrenergic receptors declines by 50% with age, as does the ability of β adrenergic agonists to activate adenylyl cyclase in senescent compared with young rats (5). CGP-12177 is both a specific β_3 adrenergic agonist and a β_1 adrenergic antagonist (7). Using this compound, we have demonstrated that the β_3 adrenergic–stimulated increase in body temperature, whole body O_2 consumption, and BAT mitochondrial GDP binding, as well as the induction of uncoupling protein mRNA, is diminished in senescent compared with young rats (3, 8). Collectively, these data suggest that decreases in the number of β_3 adrenergic receptors or impaired β_3 adrenergic activation of adenylyl cyclase may be contributing to the attenuated sympathetically activated thermogenesis with age.

In addition to the β_3 adrenergic subtype, the β_1 adrenergic receptor subtype is also present in BAT, and both the β_1 and the β_3 adrenergic receptors are coupled to the stimulatory G protein and the subsequent activation of adenylyl cyclase (9). Activation of the β_3 adrenergic subtype is predominantly responsible for thermogenic responses, whereas activation of the β_1 adrenergic subtype has been linked to cell proliferation (9, 10). Therefore, it is necessary first to quantify β_3 subtype activation with age in order to correlate β adrenergic receptor changes with the diminished BAT thermogenesis with age. Although specific subtype quantification of β_3 adrenergic receptors is hampered by the limited availability of a specific high-affinity radioligand (11), the relative contribution of β_1 and β_3 adrenergic receptors in the activation of adenylyl cyclase can be determined by resolution of dose-response activation curves into the respective β_1 and β_3 adrenergic receptor-stimulated components (12). To this end, we examined the dose-response activation of adenylyl cyclase by both the nonspecific β adrenergic agonist, isoproterenol, and the specific β_3 adrenergic agonist, BRL 37344, in BAT membranes from 4- and 24-month-old rats. Further, we compared these result to the steady-state levels of β_1 and β_3 adrenergic receptor mRNA in BAT from young and senescent rats.

Materials and Methods

Animals. Male F-344 NNia rats of 4 and 24 months of age were obtained from Harlan Sprague-Dawley (Indianapolis, IN) under contract with the National Institute on Aging. Upon arrival, rats were examined and remained in quarantine for 1 week. Animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals*. Rats were housed individually in microisolated cages and maintained on Purina Rat Chow *ad libitum* with a 12:12-hr light:dark cycle (06:00 to 18:00 hr). Experiments were begun 60–90 min after the beginning of the light cycle. Ambient temperature was 26°C.

Chemicals. BRL 37344 was a gift of SmithKline Beecham (London, England). BRL 37344 was prepared in pyrogen-free saline. Forskolin was obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

BAT Membranes. At the time of death, the circulatory system was perfused with 20 ml of cold saline, and interscapular BAT was excised and trimmed of visible white fat. To prepare the membranes, BAT was suspended in 250 m*M* sucrose, 1 m*M* $MgCl_2$, 5 m*M* Tris, pH 7.4, and a protease inhibitor cocktail consisting of (in μ*M*) 1 lupetin, 100 benzamidine, and 100 phenylmethylsufonyl fluoride (PMSF). BAT was finely minced with a tissue chopper, disrupted in a tissuemizer for 30 sec and homogenized with 10 strokes of a motor-driven pestle. The homogenate was passed through four layers of cheesecloth and centrifuged at 48,000*g* for 20 min at 4°C. The pellet was resuspended in (in m*M*) 18 $MgCl_2$, 0.08 ascorbic acid, and 50 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4. Membranes were immediately used in the adenylyl cyclase assay.

Adenylyl Cyclase Assay. Adenylyl cyclase activity was assayed by the conversion of ATP into adenosine 3′,5′-cyclic monophosphate (cAMP) in the presence of a methylxanthine and an ATP regeneration system as previously described (13). cAMP was quantified by radioimmunoassay. Protein was determined by the method of Bradford (14).

Data Analysis. Agonist-stimulated adenylyl cyclase activation curves were resolved by a least squares computer modeling method using Inplot (Graph Pad Software, San Diego, CA). *F* tests were used to determine whether one- or two-component models better represented the data. Average estimates of affinity constants were expressed as geometric means. For this purpose, the arithmetic mean of the logarithmic transform of individual estimates of the affinity constants was calculated, and its antilogarithmic transform was used as the geometric mean (15). Statistical significance was determined by one-way analysis of variance (ANOVA).

β_1AR and β_3AR mRNA Levels. β_1 and β_3 adrenergic receptor mRNAs were measured simultaneously in the same sample with a RNase protection assay (RNase Protection Assay System; Promega, Madison, WI). Radioactive cRNA probes were transcribed *in vitro* with [^{32}P]CTP using the T7 promoter (Riboprobe Transcription System, Promega). The β_1 receptor cDNA (p119) and the β_3 receptor cDNA (p110) were provided by James Granneman (16), and were linearized at the internal *Rsa*I site and the *Hin*dIII site in the vector, respectively. BAT RNA (40 μg) was coprecip-

itated with 3×10^4 cpm of each [^{32}P]-labeled probe. Samples were resuspended in 20 μl hybridization buffer containing 80% formamide, 200 m*M* NaAcetate, 1 m*M* EDTA, and 40 m*M* piperazine-N,N′-bis-(2-ethane-sulfonic acid), pH 6.4, and hybridized at 55°C for 16–18 hr. Samples were diluted in 10 volumes of 200 m*M* NaAcetate, 5 m*M* EDTA, and 10 m*M* Tris, pH 7.5, and 10 units RNase were added to each sample. Digestions were incubated for 1 hr at room temperature and the reaction terminated with 20 μl 10% SDS and 1 mg/ml tRNA, followed by ethanol precipitation. Precipitates were resuspended in 1 m*M* EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS, and 80% formamide. The [^{32}P]-labeled RNA probes that were protected from RNase digestion were electrophoretically resolved on a denaturing polyacrylamide gel containing 8 *M* urea. The gels were dried, exposed to a phosphoimaging screen, scanned by a Phospho Imager (Molecular Dynamic, Sunnyvale, CA) and analyzed by Image Quant Software (Molecular Dynamics). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were determined by slot blot analysis as described previously (17).

Results

β_1AR- and β_3AR-Mediated Adenylyl Cyclase Activity. Adenylyl cyclase activity was assessed in BAT membranes from young and senescent rats. Maximal stimulation of adenylyl cyclase by both the β_3AR selective agonist, BRL 37344, and the non-selective agonist, isoproterenol, was nearly 2-fold less in BAT membranes from the senescent than in those from the young rat (Table I). As previously reported, the wet weight of the BAT and the amount of isolated membrane protein was slightly less in senescent than in the young rats despite an increase in rat weight (8, 18). Thus, the decrease with age in adenylyl cyclase activity was exacerbated when the total activity per BAT tissue was considered (data not shown). In addition to the reduced β adrenergic agonist–stimulated adenylyl cyclase activity with age, glucagon, another thermogenic agent, demonstrated dramatically less stimulation in the senescent rats to a level just above GTP stimulation (Table I). In addition, GTP-stimulated adenylyl cyclase activity demonstrated a 37% decrease with age. Similarly, the postreceptor activators, NaF and forskolin, also stimulated adenylyl cyclase by 32% and 44% less, respectively, in BAT membranes from senescent rats compared with young rats (Table I).

Table I. Adenylyl Cyclase Activity in Brown Adipose Tissue from Young and Senescent Rats

Stimulator	Adenylyl cyclase activity (pmol cAMP/min/mg protein)	
	Young (4 m)	Senescent (24 m)
None	21.3 ± 2.7	14.2 ± 1.8
GTP (10 μ*M*)	37.2 ± 4.0	23.5 ± 1.7[a]
BRL 37344 (100 μ*M*)	222 ± 29	118 ± 10[a]
Isoproterenol (1 m*M*)	179 ± 22	104 ± 5[a]
Glucagon (100 μ*M*)	146 ± 20	40 ± 3[a]
NaF (10 m*M*)	204 ± 22	138 ± 15[a]
Forskolin (100 μ*M*)	171 ± 26	96 ± 9[a]

Note. Values represent means ± SEM of five to seven rats per group. BRL 37344-, isoproterenol-, and glucagon-stimulated activity represents activity in the presence of 10 μ*M* GTP.
[a] $P < 0.025$ for difference with age by one-way ANOVA.

The stimulation of adenylyl cyclase by isoproterenol and BRL 37344 was investigated further by determining dose response activation curves. Isoproterenol activates adenylyl cyclase in BAT by interacting with both β_1ARs and β_3ARs as suggested by the biphasic nature of the activation curve (Fig. 1). Granneman (12) has demonstrated that the β_1ARs mediate the high-affinity component whereas β_3ARs mediate the low affinity component. The activation curves were analyzed by nonlinear regression modeling, and the analysis indicated that the activation curves were best represented by two sites, with the β_1ARs and β_3ARs each mediating approximately 50% of the total isoproterenol-stimulated activity (Fig. 1 and Table II). The calculated values of the V_{max} for β_1AR- and β_3AR-mediated activation in BAT membranes from senescent rats were diminished by 48% and 38%, respectively, compared with V_{max} values from young rats (Table II). The concentration for half-maximal stimulation, K_{act}, for the β_1AR-mediated activation was 6-fold less (higher affinity) in the membranes from senescent compared with young rats, whereas the K_{act} for the β_3AR-mediated activation was not significantly different with age (Table II).

The BRL dose-response activation curves were also subjected to computer modeling, and the analysis indicated that the data was best represented by two sites (Fig. 2). Although BRL 37344 is a selective β_3 adrenergic agonist, it has demonstrated low-affinity interaction with other βAR subtypes (11, 19). The β_3AR mediated 67% and 80% of the total adenylyl cyclase activity, respectively, in BAT from young and senescent rats (Fig. 2). The calculated value for the V_{max} of the β_3AR-mediated activation was 37% less in the senescent compared with the young rats, an amount equal to the reduction with age in the β_3AR-mediated, isoproterenol-stimulated activity (Table II).

β_1AR and β_3AR mRNA Levels. The levels of β_1AR and β_3AR mRNA were determined simultaneously in total RNA from BAT from young and senescent rats. Northern analysis indicated the β_1AR probe hybridizes to single mRNA species of 3.1 kb in BAT and brain, and the β_3AR probe hybridizes to three major bands at 3.8, 2.8, and 2.4 kb in BAT (data not shown). The relative abundance of β_3AR mRNA

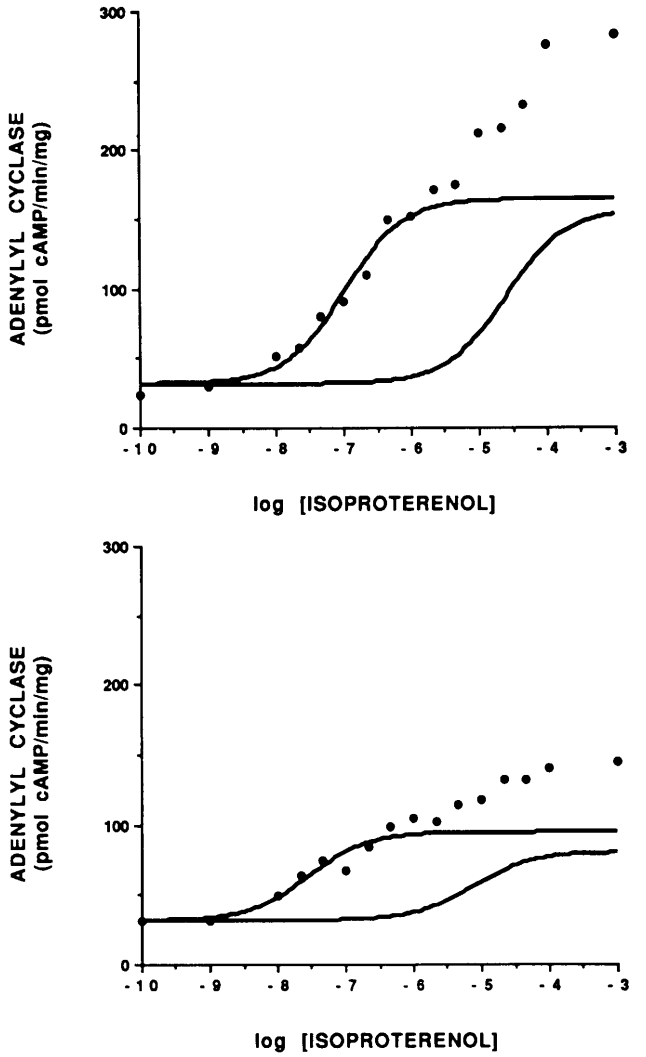

Figure 1. Isoproterenol dose-response activation of adenylyl cyclase in BAT membranes from young (top) and senescent (bottom) rats. Activation curves were resolved by least squares analysis into two components: high-affinity, β_1 adrenergic receptor activation (upper curve) and low-affinity, β_3 adrenergic receptor activation (lower curve). See Table II for K_{act} and V_{max} values. Data is a representative activation curve (out of 6) from a single young and a single old rat.

was approximately 15 times that of β_1AR mRNA in both the young and senescent rats (Table III). The steady-state levels of both β_1AR and β_3AR mRNA decreased by approximately 2-fold in senescent compared with young rats (Table III). The levels of β_1AR and β_3AR mRNA were normalized to the amount of GAPDH mRNA present in each sample. The steady-state levels of GAPDH mRNA did not change with age (32.5 ± 5.4 vs 29.4 ± 3.3, arbitrary units).

Discussion

The β_3 adrenergic receptor is believed to be the prevalent β_3 adrenergic subtype present in rodent BAT (11). In addition to this subtype, there are coexisting β_1 and β_2 adrenergic receptors (9), however, the exact role of these various subtypes in BAT is unclear. Although neither the β_1 nor the β_2 adrenergic subtype appears to be coupled to thermogenesis in BAT from rodents, the β_1 adrenergic subtype plays an important role in cell proliferation especially in the immature adipocyte (9, 10). Both the β_1 and the β_3 adrenergic subtypes are coupled to adenylyl cyclase activation *via* the stimulatory G protein. Moreover, Granneman demonstrated that both subtypes activate the same pool of adenylyl cyclase in isolated white adipocytes (12). A similar conclusion is suggested by the data presented in this report. If two receptors stimulate a common adenylyl cyclase, then stimulation by both would not be additive (20). The maximum extent of isoproterenol stimulation of adenylyl cyclase, representing activity mediated by both the β_1 and the β_3 subtype was similar to the stimulation by BRL 37344, representing activity mediated by only the β_3 adrenergic

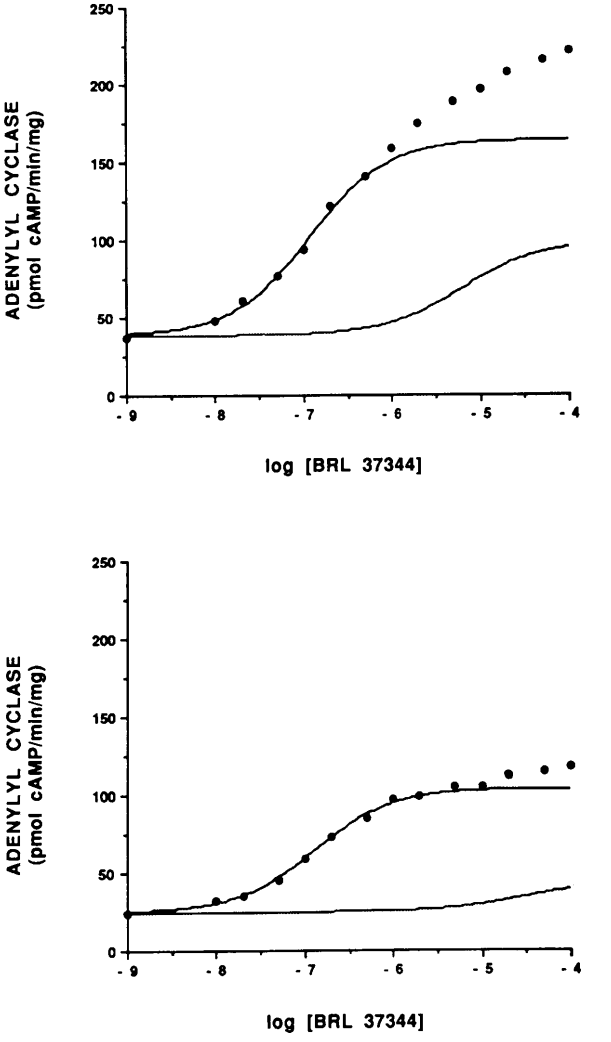

Figure 2. BRL 37344 dose-response activation of adenylyl cyclase in BAT membranes from young (top) and senescent (bottom) rats. Activation curves were resolved by least squares analysis into two components: high-affinity (upper curve) and low-affinity (lower curve) activation. See Table II for K_{act} and V_{max} values. Data represents the composite of activation curves from seven rats in each age group.

Table II. The β_1 and β_3 Adrenergic Receptor Components of Adenylyl Cyclase Activation in BAT with Age

	Young (4 m)		Senescent (24 m)	
	β_1	β_3	β_1	β_3
Isoproterenol				
V_{max} (pmol cAMP/min/mg)	87 ± 14	91 ± 12	45 ± 5[a]	57 ± 4[a]
K_{act} (μM)	0.10 ± 0.09	13 ± 11	0.016 ± 0.004[a]	3.1 ± 2.2
% total	48 ± 14	52 ± 4	43 ± 4	57 ± 4
BRL 37344				
V_{max} (pmol cAMP/min/mg)		126 ± 20		79 ± 8[a]
K_{act}		0.12 ± 0.02		0.13 ± 0.01

Note. Values represent means ± SEM of five to seven rats per group. V_{max} and K_{act} were determined from two-site least squares analysis of isoproterenol and BRL 37344 adenylyl cyclase activation curves. V_{max} represents activity above basal activity.
[a] $P < 0.01$ for difference with age by one-way ANOVA.

receptor. Moreover, the level of activation by these β adrenergic agonists was the same as NaF and forskolin, indicating that the activation was not limited by available stimulatory G protein and that, most likely, all the cyclase available to these receptors was activated. A similar pattern of stimulation of adenylyl cyclase was observed in BAT from senescent rats, suggesting at both ages the β_1 and β_3 adrenergic receptors are coupled to the same pool of adenylyl cyclase. Although the potency of isoproterenol for the β_1 subtype was 130–200 times greater than for the β_3 subtype, with maximum isoproterenol stimulation, both receptors contributed equally to the activation of adenylyl cyclase in young and senescent rats.

Sympathetically activated thermogenesis in BAT declines with age (1, 3). The β_3 adrenergic receptor is the principal mediator of thermogenesis in the mature adipocyte from young rats (9), and the β_3 adrenergic agonist stimulation of thermogenesis, including oxygen consumption, body temperature, and mitochondrial GDP binding, as well as the induction of uncoupling protein mRNA, is impaired with age (3, 8). In contrast to these findings, when forskolin, which directly activates adenylyl cyclase, was administered to young and senescent rats, we found that the increase in whole body oxygen consumption, the increase in GDP binding and the induction of uncoupling protein mRNA were unchanged with age (21).

Collectively, these data indicate that β_3 adrenergic-mediated thermogenesis in BAT is impaired with age but that postreceptor-mediated thermogenesis is maintained with age. These findings suggest that the reduced β_3 adrenergic–mediated thermogenesis in BAT with age is a result of deficient numbers of β_3 adrenergic receptors or impaired β_3 adrenergic receptor coupling to adenylyl cyclase with age or both. Supporting this is our previous report indicating that the total number of β adrenergic receptors declines by 50% with age in BAT (5), and the recent report in white adipose tissue indicating a decrease with age in β_1 and β_3 adrenergic mRNA levels (22).

Although the specific subtype quantification of β_1 and β_3 adrenergic receptors is hampered by the limited availability of a specific high-affinity radioligand (11), in the present report the relative contribution of β_1 and β_3 adrenergic receptors in the activation of adenylyl cyclase with age was determined by two-site regression analysis of isoproterenol activation curves compared with BRL 37344 activation curves. Maximum activation by both isoproterenol and BRL 37344 was decreased by approximately 50% in senescent compared with the young rats. Analysis of the activation curves indicated the activity mediated by both the β_1 and the β_3 adrenergic subtypes also declined by 50% with age. Parallel to this decrease with age, the steady-stage mRNA levels of β_1 and β_3 adrenergic receptors declined by 50% in BAT from senescent compared with young rats. The relative abundance of β_3 adrenergic receptor mRNA was 15 times greater than the abundance of β_1 adrenergic mRNA in both the young and senescent rats. This is a considerably higher ratio than the 2:1 proportion of β_3 to β_1 adrenergic receptor as assessed by radioligand assays (23).

Our previous studies indicated the decline in isoproterenol-stimulated adenylyl cyclase activity and β_3

Table III. Steady-State Levels of β_1AR and β_3AR mRNA from BAT of Young and Senescent Rats

Receptor	mRNA levels (arbitrary units)	
	Young (4 m)	Senescent (24 m)
β_3AR	100 ± 12	54 ± 5[a]
β_1AR	8.01 ± 1.15	3.94 ± 0.74[a]
β_3AR/β_1AR	14.2 ± 2.1	16.4 ± 4.2

Note. β_1AR and β_3AR mRNA levels are expressed as arbitrary units per μg RNA and normalized to the amount of GAPDH mRNA present in each sample. Values represent means ± SEM of nine young and five senescent rats.
[a] $P < 0.02$ (β_3AR) or $P < 0.03$ (β_1AR) for difference with age by one-way ANOVA.

adrenergic agonist-stimulated thermogenesis was apparent in both adult (12- or 18-month-old) and senescent (24-month-old) rats (3, 5). The present study was limited to two ages, young and senescent, and thus cannot determine whether the decline in β adrenergic subtype activation of adenylyl cyclase occurs in adult compared with young animals.

The above data, taken alone, suggest diminished receptor number could account for the impaired β adrenergic signal transduction in BAT from senescent rats. However, maximum stimulation of adenylyl cyclase by NaF and forskolin was also diminished with age by the same amount as β adrenergic stimulation, suggesting that the activation with age may be limited by the amount of adenylyl cyclase catalytic unit rather than receptor number. In contrast, the glucagon-stimulated adenylyl cyclase activity was diminished by more than 70%, suggesting glucagon receptor number or coupling to adenylyl cyclase is the causative factor in the glucagon-stimulated adenylyl cyclase decrease with age. These data are similar to those reported for a number of organs where the efficacy for β adrenergic stimulation of adenylyl cyclase declines with age. For the most part, the decrease is associated with a decline in forskolin-stimulated activity (24), and in one case in the rat myocardium the decrease has been attributed to the decline in the number of adenylyl cyclase catalytic units with age (25).

In summary, the diminished β_3 adrenergic–stimulated BAT thermogenesis with age may be a combination of both reduced ability of β_3 adrenergic receptors to activate adenylyl cyclase and decreased amount of adenylyl cyclase catalytic units with age.

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