Gabaldón, Annette M., Roger B. McDonald, and Barbara A. Horwitz.

Effects of age, gender, and senescence on β-adrenergic responses of isolated F344 rat brown adipocytes in vitro. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E726–E736, 1998.—We previously reported greater age-related attenuation of cold-induced thermoregulation and brown adipose tissue thermogenic capacity in male vs. female F344 rats. With onset of the rapid weight loss that occurs near the end of the lifespan, this age-related attenuation becomes severe. We refer to this “end-of-life” physiological state of older rats as senescence. Here, we measured oxygen consumption of isolated brown adipocytes and found no age (6 vs. 12 vs. 26 mo) or gender effects on maximal norepinephrine (NE)- or CL-316,243 (β3-adrenergic agonist)-induced responses. In contrast, brown adipocytes from 22- to 26-mo-old senescent rats (males and females) consumed 51–60% less oxygen during maximal stimulation with NE and CL-316,243 than did cells from 26-mo-old presenescent rats. This attenuation was associated with lower (65–72%) uncoupling protein 1 concentrations but no alterations in NE-induced cAMP levels or lipolysis. Our data indicate that senescence, but not chronological age, significantly impacts NE-/β3-mediated thermogenesis of isolated brown adipocytes and that this effect involves altered mitochondrial rather than altered membrane or cytosolic events.

The reduced cold tolerance of senescent vs. presenescent rats was associated with significantly diminished BAT thermogenic capacity, as manifested by substantially lower amounts of total tissue protein (35% lower) and total UCP-1 (48% lower) (14). The development of very severe cold-induced hypothermia in senescent rats is not due to decreased food intake or body weight loss per se, as indicated by the absence of severe cold-induced hypothermia in 25- to 26-mo-old weight-stable rats that were food restricted to reduce their body weight by the same amount (14). Thus rapid weight loss appears to be a marker of this altered physiological state rather than a direct cause of it.

This investigation tested the hypothesis that the differences in cold-induced thermoregulation and BAT thermogenic capacity associated with chronological age, gender, and senescence involve alterations in thermogenesis of brown adipocytes themselves. To test this hypothesis, we measured oxygen consumption of isolated brown adipocytes in response to NE and CL-316,243, a highly selective β3-adrenergic agonist (1), both of which stimulate BAT thermogenesis. In experiment 1 we studied weight-stable, aged rats to evaluate the effects of chronological aging; in experiment 2 we studied senescent rats. In the latter, we also measured...
NE- and forskolin-stimulated cAMP levels and lipolysis, as well as the UCP-1 content of the isolated brown adipocytes, because NE-stimulated thermogenesis in BAT involves activation of the adenyl cyclase-cAMP second messenger pathway and lipolysis.

**MATERIALS AND METHODS**

**Animals and Animal Care**

Male and female F344 rats, aged 6, 12, and 22–23 mo, were obtained from the National Institute on Aging colony maintained by Harlan Sprague Dawley Laboratory (Indianapolis, IN). On arrival, rats were housed individually in hanging wire-bottom cages (20 × 25 × 18 cm) and maintained at 25–26°C and 50% humidity on a 12:12-h light-dark cycle (lights on at 0600, off at 1800). The 22- to 23-mo-old rats were placed into a laminar flow unit (Duo-Flo, Lab Products, Maywood, NJ) that provides clean air by circulating air through high-efficiency particle filters. NIH-31 laboratory chow (Teklad Research Diets, Indianapolis, IN) and distilled water were provided ad libitum. The 6- and 12-mo-old rats were maintained in our facility for 2 wk before experimentation. The 22- to 23-mo-old rats were maintained up to a maximum age of 26 mo, and their body weights were measured every 2–3 days during this time. At the time they were killed, the animals were visually inspected. There was no gross pathology significant enough to warrant exclusion of any of the old rats.

**Experimental Design**

Older rats were subdivided into two groups on the basis of body weight stability. Rats that maintained a stable body weight during the experimental period are referred to as presenescence; rats that exhibited rapid weight loss are referred to as senescent. The presenescence rats were killed at 26 mo of age for isolation of brown adipocytes; the senescent rats were killed between 22 and 26 mo, typically after a minimum of 4 days of weight loss. In the study on brown adipocyte oxygen consumption, the percentage of weight loss for senescence males (n = 14) was 17.0 ± 1.1% (SE), with a range of 11.0–27.1%, and for senescent females (n = 9) it was 15.1 ± 0.8%, with a range of 11.5–19.0%. To evaluate the influence of weight loss on oxygen consumption of brown adipocytes, cells were isolated from a group of older presenescence rats induced to lose weight via food restriction. Male F344 rats aged 25–26 mo (n = 10) were given daily an amount of food equal to 50–60% of their ad libitum consumption until a mean weight loss of 17.1 ± 1.7% with a range of 11.3–25.1% was observed. Rats selected for this experiment had maintained stable body weights for ≥1 mo before initiation of food restriction. cAMP levels and lipolytic activity of brown adipocytes were evaluated in a second group of presenescence (n = 6–8) and senescent (n = 5–6) male F344 rats. Cells were isolated from senescent rats at a mean percentage weight loss of 19.1 ± 2.3% with a range of 9.1–27.7%. The UCP-1 content of brown adipocytes was determined in a third group of presenescence (n = 8) and senescent (n = 13) male F344 rats. Cells were isolated from senescent rats at a mean percentage weight loss of 18.7 ± 1.2% with a range of 12.0–25.6%.

**Isolation of Brown Adipocytes**

Brown adipocytes were isolated from brown fat of each rat by modification of the method of O'Donnell and Horwitz (16). Rats were killed by decapitation between 0900 and 1030, and BAT from the interscapular, subscapular, cervical, axillary, and dorsal aortic regions was rapidly removed, trimmed of any adhering muscle and white fat, weighed, and then minced with scissors in Krebs-Ringer bicarbonate buffer (KRB; with BSA, KRBA; see Buffers and Chemicals). The tissue pieces were rinsed over a silk filter with fresh buffer, transferred to a 25-ml Nalgene flask containing KRBA (1 ml/100 mg tissue) plus collagenase (2 mg/ml) and trypsin inhibitor (0.3 mg/ml), and preincubated at 37°C for 18 min in a shaking [100 counts/min (cpm)] water bath under an atmosphere of 95% O2-5% CO2. The resulting suspension was poured through a silk filter, and this initial filtrate was discarded. The tissue pieces were resuspended in fresh KRB, as just described but with 1 mg/ml collagenase and 0.15 mg/ml trypsin inhibitor, and incubated for 32 min longer. The suspension was poured through silk cloth, and the isolated cells were collected in a 50-ml plastic centrifuge tube. Additional brown adipocytes were obtained from the remaining tissue fragments by gently expressing the cells through the silk filter. For this, the square of silk cloth was gathered at the edges, forming a pouch around the tissue pieces, and immersed in 3 ml of fresh KRBA. Cells were expressed by applying gentle pressure to the silk bag with the aid of a siliconized glass stirring rod. After 3–4 strokes, the buffer containing expressed cells was poured through another silk filter and collected in a second 50-ml plastic centrifuge tube. This process of expression and collection was repeated several times using fresh buffer until there was little remaining tissue.

Brown adipocytes isolated for measurement of oxygen consumption, cAMP, and lipolysis were washed twice by flotation in KRBA buffer for ~35 min at room temperature; total isolation time was typically 3 h, and measurements were begun immediately after this. Brown adipocytes isolated for measurement of UCP-1 content were washed twice by centrifugation (10 min at 100 g) in BSA-free KRB buffer and were stored at −70°C until the day of the assay. Mature brown adipocytes were visually identified by their multilocular appearance. Cell counts were performed with an improved Neubauer hemacytometer using trypan blue stain (0.4%) for visualization and exclusion of broken cells. The percentage of broken cells was typically 23–30% in all groups. Whereas the total cell yield from presenescence old rats did not differ from that of the 6-mo-old animals, the yield from senescent rats appeared to be lower. [For example, the total number of brown adipocytes isolated for measurement of oxygen consumption averaged (± SE) as follows (×10⁶): for males: 6 mo, 10.9 ± 1.5; 12 mo, 7.9 ± 1.0; old presenescence, 9.2 ± 0.9; senescent, 6.7 ± 1.2; and for females: 6 mo, 12.8 ± 1.5; 12 mo, 8.1 ± 2.0; old presenescence, 12.5 ± 1.5; senescent, 5.9 ± 1.3].

**Isolated Brown Adipocyte Measurements**

Oxygen consumption. Oxygen consumption was measured polarographically with a Clark-type probe linked to a Gilson Oxygraph oxygen monitoring system (model 5/6H). Brown adipocytes were suspended in KRBA buffer (30,000–50,000 intact cells/ml) and added to a magnetically stirred 1.8-ml cell chamber thermostated at 37°C. Cells were incubated for 10–15 min to measure the resting respiratory rate. After this, different agonist additions (2.5 μl volumes) were made with a Hamilton syringe through a small hole in the chamber lid. Respiratory responses to a single maximal concentration (25 μM) of CL-316,243 and to different NE concentrations were evaluated for each rat. The concentration-response curves for NE were generated by successively adding 10-fold increasing amounts of NE to the chamber according to the method of Udellius et al. (23). The response to each addition was
recorded until a new stable rate of respiration was reached (−4−5 min), and then the next addition was made. In preliminary experiments, we observed no difference in these rates of respiration compared with responses of cells that were exposed to a single specific concentration of NE, indicating no desensitization of the response with this successive addition of agonist. Measurements were typically performed on three aliquots of cells over three ranges of NE concentration (in µM): 0.001−10, 0.0025−2.5, and 0.005−5; these data were combined to generate one single concentration−response curve per rat. Lines were drawn and maximal oxygen consumption rates (V_{max}) and 50% effective concentration (EC_{50}) values were calculated by reiterative computerized fitting of the data points to a Michaelis-Menten type equation, as described by Unelius et al. (23) by use of Delta Graph (DeltaPoint) (see Fig. 1). Oxygen consumption rates were calculated on the basis of 750 nmol O_{2}/ml KRBA equilibrated with 95% O_{2}-5% CO_{2} at 37°C based on the equation (nmol O_{2}/ml) = [%O_{2} of gas mixture × (P_{barometric} − P_{water vapor}) × solubility of O_{2} in plasma]/760 × molar volume of gas × 100, where solubility was taken as 0.0215 ml O_{2}/ml plasma at 760 mmHg at 37°C, according to Fasico and Chiodi (3).

Lipolysis. Lipolytic activity of brown adipocytes was measured as the amount of glycerol released into the incubation medium by modification of the method of Hamilton and Horvitz (8). Aliquots of the cell suspension (40,000−50,000 intact brown adipocytes/200 µl) were added to 3-ml plastic vials containing KRBA buffer and agonist (NE or forskolin) to give the desired final concentration in a total volume of 1 ml. The vials were gassed for 5−7 s with 95% O_{2}-5% CO_{2} capped tight, and then incubated for 30 min in a 37°C shaking (60 cpm) water bath with periodic mixing by hand every 10−15 min. Our preliminary studies indicated that resting and maximal NE-induced glycerol release was linear for ≥60 min of incubation at 37°C. After incubation, vials were placed on ice for ≥30 min to allow the adipocytes to separate from the buffer. An aliquot (500 µl) of the infranatant was then removed and transferred to a 1.5-ml microfuge tube. These were placed in a boiling water bath for 10 min, returned to the ice for several minutes, and then centrifuged in the cold (45 min at 10,000 g). The glycerol content of the deproteinized supernatants was determined enzymatically with a Sigma Chemical kit [Triglyceride (GPO-Trinder) no. 337].

**cAMP levels.** cAMP levels were measured by modification of the method of Svartengren et al. (22). Aliquots of the cell suspension (20,000−25,000 intact brown adipocytes/100 µl) were added to 1.5-ml microfuge tubes containing KRBA buffer in a total volume of 0.5 ml and were preincubated for 3 min at 37°C. cAMP formation was initiated by addition of NE or forskolin to give the desired final concentration in a total volume of 0.5 ml. The samples were returned to the 37°C shaking (100 cpm) water bath, and the reaction was halted at 3 min by addition of 1 ml ice-cold 95% ethanol. (Our preliminary studies indicated peak NE-induced cAMP levels at 3 min under these experimental conditions). The tubes were vortexed vigorously, placed on ice for ≥20 min, and then centrifuged in the cold (10 min at 6,000 g). The supernatants were removed and transferred to 6-ml polypropylene tubes, and the remaining precipitates were washed once by addition of 1 ml ice-cold 65% ethanol as described above. The two supernatants were combined and evaporated overnight under vacuum at 60°C. The dried extracts were stored at −70°C until subsequent analysis. On the day of each assay, dried extracts were solubilized in a suitable volume of assay buffer, and cAMP was measured by enzyme immunoassay of nonacetylated cAMP samples (Amersham kit RPN 225). Phosphodiesterase activity was not inhibited during the incubations.

Uncoupling protein 1 (UCP-1). The UCP-1 content of brown adipocytes was determined by immunoassay by use of a modification of the method of Lean et al. (11). On the day of each assay, cells were thawed and sonicated on ice, diluted to a concentration of 10.7 µg total protein per well, and separated by SDS-PAGE. After electrophoresis, protein bands were electrically transferred to nitrocellulose membranes, which were then blocked and probed using sera from rabbits immunized against rat UCP, purified by the method of Lin and Klingenberg (12). The UCP-probed band was visualized by a color reaction with goat anti-rabbit antibody coupled to alkaline phosphatase with an assay kit (Bio-Rad, Richmond, CA). Bands were quantified via scanning densitometry, and experimental samples were compared with values obtained for purified rat UCP-1 standards. The anti-rat UCP sera did not react with liver or muscle preparations, indicating little or no cross-reactivity with UCP-2 or UCP-3, recently identified variants of brown adipocyte-specific UCP-1 (2, 4, 7).

**Buffer and Chemicals**

Kreb's-Ringer bicarbonate buffer (KRB) contained (in mM): 118 NaCl, 4.7 KCl, 1.25 CaCl_{2}, 1.2 KH_{2}PO_{4}, 1.2 MgSO_{4}, and 24.9 NaHCO_{3}. KRB solutions were prepared weekly from refrigerated concentrated stock solutions and modified on the day of each experiment with 4% BSA, 10 mM glucose, 250 U/ml penicillin, and 250 µg/ml streptomycin. The resulting KRB buffer was then filtered (0.22 µm filter, Corning) and adjusted to pH 7.4 by gassing with 95% O_{2}-5% CO_{2}.

Penicillin/streptomycin solution was purchased from Grand Island Biochemical (Grand Island, NY); collagenase (crude bacterial, type CLS-1) was from Worthington Biochemical (Freehold, NJ); NE (L-arterenol bitartrate), forskolin, trypsin inhibitor (from soybean, type 1-S), and BSA (fatty acid content <0.005%) were from Sigma Chemical (St. Louis, MO); and CL-316,243 was a gift from American Cyanamid. Adrenergic agonists were solubilized in a 1 mg/ml solution of ascorbic acid to prevent oxidation and stored in 15-µl aliquots at −70°C until the day of the experiment.

**Statistics**

The data were analyzed using multifactorial analysis of variance (ANOVA) or unpaired two-tailed Student’s t-test where appropriate. When a significant main effect was found by ANOVA, a protected Fisher least significant difference post hoc test was used to evaluate differences between specific groups. Differences were considered significant at P ≤ 0.05. Values are means ± SE.

**RESULTS**

Experiment 1. Effects of Chronological Age and Gender on Brown Adipocyte Oxygen Consumption

Body mass and BAT mass. As expected, there was a significant effect of gender and age on body mass, with male rats weighing more than females, and 6-mo-old rats weighing less than 12- and 26-mo-old rats (Table 1). The fact that body mass increased significantly between 12 and 26 mo of age in females but not in males resulted in a significant interaction of age and gender. There was no gender difference in the total mass of interscapular BAT (mg), but there was an effect of age that reflected higher values in 12- and 26- vs. 6-mo-old rats. In contrast, analysis of body mass-adjusted data revealed no age effect but significantly more interscapular BAT (mg/g body mass) in females vs. males. At age
ther age nor gender had a significant effect on response was obtained for each cell preparation. NE-induced oxygen consumption increased significantly in all groups, with the responses being comparable to those induced by maximal NE, being slightly greater in males compared with females. This was influenced mainly by values for 6- and 12-mo-old rats (~2 times lower EC50 values in males compared with females), with no significant difference observed between 26-mo-old males and females (0.08 ± 0.01 vs. 0.11 ± 0.01 µM).

CL-316,243 stimulation of oxygen consumption. Figure 2 shows the effect of CL-316,243 on respiration rate per age and per gender for the same brown adipocyte preparations represented in Fig. 1. There were no significant effects of age or gender on resting respiration values (range of means: 46 ± 9 to 98 ± 23 nmol O2 · min⁻¹ · 10^6 cells⁻¹). When the cells were stimulated with a single addition of a maximally stimulating concentration (25 µM) of CL-316,243, respiration increased significantly in all groups, with the responses being comparable to those induced by maximal NE concentrations. There was a main effect of gender but not age on respiration in the presence of CL-316,243.

Table 1. Body and BAT mass in F344 rats in experiment 1

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Female</th>
<th>Male</th>
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<tbody>
<tr>
<td>6 (n = 9)</td>
<td>206 ± 6A</td>
<td>359 ± 16D</td>
</tr>
<tr>
<td>12 (n = 11)</td>
<td>248 ± 5B</td>
<td>442 ± 7E</td>
</tr>
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<td>26 (n = 14)</td>
<td>294 ± 6C</td>
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</tr>
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</table>

Values are means ± SE; n, no. of rats. Within a row, means with a common letter superscript are not significantly different. Total brown adipose tissue (BAT) mass represents combined interscapular, subscapular, cervical, axillary, and dorsal aortic BAT depots from which brown adipocytes were isolated.

26 mo, this amounted to ~64% more BAT in females than in males. Total BAT mass shown is for the combined interscapular, subscapular, cervical, axillary, and dorsal aortic BAT depots from which the brown adipocytes were isolated. In general, total BAT mass (mg/g body mass) paralleled that of interscapular BAT alone, being significantly greater in females than in males at all ages.

NE-induced oxygen consumption. Figure 1 shows composite concentration-response curves per gender and per age for NE-induced respiration of brown adipocytes. Resting respiration, NE-induced Vmax, and EC50 values calculated from curves for individual rats are shown in Table 2. There were no main effects of age or gender on resting respiration values, which averaged between 50 ± 10 and 61 ± 15 nmol O2 · min⁻¹ · 10^6 cells⁻¹ for all groups except for the 6-mo-old females (27 ± 8). With the addition of NE, respiration increased in a concentration-dependent manner, and a maximal response was obtained for each cell preparation. Neither age nor gender had a significant effect on Vmax (range of means: 537 ± 32 to 654 ± 42 nmol O2 · min⁻¹ · 10^6 cells⁻¹). There was an effect of gender but not age on sensitivity (indexed by EC50) of brown adipocytes to NE, being slightly greater in males compared with females. This was influenced mainly by values for 6- and 12-mo-old rats (~2 times lower EC50 values in males compared with females), with no significant difference observed between 26-mo-old males and females (0.08 ± 0.01 vs. 0.11 ± 0.01 µM).

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![Fig. 1](http://ajpendo.physiology.org/)

Table 2. Correlation coefficients for curve fits were

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Fig. 1. Comparison of composite concentration-response curves for norepinephrine (NE)-induced oxygen consumption of isolated brown adipocytes from female (A, B, and C) and male (D, E, and F) F344 rats aged 6 (A and D), 12 (B and E), and 26 mo (C and F) (experiment 1). Results are means ± SE for no. (n) of rats in parentheses; where not shown, SE is smaller than the size of symbol. Values are expressed as increase over resting respiratory rate (see Table 2 for resting levels). Curves were drawn by reiterative computerized fitting of data points to a Michaelis-Menten type equation as described by Unelius et al. (23) by use of Delta Graph (DeltaPoint): 

\[ V_A = \frac{V_{\text{max}} \cdot [A]}{[A] + EC_{50}} \]

where A is agonist concentration, V_A is rate of respiration at that concentration, Vmax is maximal increase above resting respiratory rate, and EC50 is 50% effective concentration. Calculated Vmax and EC50 values are shown in Table 2. Correlation coefficients for curve fits were ≥0.90.
Table 2. Resting respiration, NE-induced $V_{\text{max}}$, and $EC_{50}$ of isolated brown adipocytes from F344 rats in experiment 1

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Resting Respiration, nmol O$_2$·min$^{-1}$·10$^6$ cells$^{-1}$</th>
<th>$V_{\text{max}}$, nmol O$_2$·min$^{-1}$·10$^6$ cells$^{-1}$</th>
<th>$EC_{50}$, µM</th>
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<tbody>
<tr>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>6</td>
<td>27 ± 8</td>
<td>61 ± 15</td>
<td>597 ± 48</td>
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<td></td>
<td>0.11 ± 0.03$^a$</td>
<td>0.04 ± 0.01$^a$</td>
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<tr>
<td>12</td>
<td>50 ± 10</td>
<td>60 ± 12</td>
<td>537 ± 32</td>
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<td></td>
<td>0.10 ± 0.02$^a$</td>
<td>0.05 ± 0.01$^a$</td>
<td></td>
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<tr>
<td>26</td>
<td>54 ± 10</td>
<td>58 ± 8</td>
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<tr>
<td></td>
<td>0.11 ± 0.01$^a$</td>
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Values are means ± SE; nos. of rats are shown in parentheses.

$V_{\text{max}}$, maximal respiration; $EC_{50}$, 50% effective concentration. For each variable, means with no letter or those sharing a common letter superscript are not significantly different. Results are derived from experiments summarized in Fig. 1 but are based on evaluation of concentration-response curves for individual rats.

that reflected moderately higher rates in males than in females.

Experiment 2. Effect of Senescence on Brown Adipocyte Oxygen Consumption, cAMP, Lipolysis, and UCP-1

Oxygen consumption. SPONTANEOUS BODY WEIGHT LOSS. The age at which spontaneous body weight loss began was similar in males and females: males = 24.3 ± 0.3 (22.4–25.8) mo and females = 24.4 ± 0.3 (23.3–25.8) mo. The average rate of weight loss (% of baseline body weight/day) varied among individuals, with males ranging from 0.45 to 2.73% and females ranging from 0.61 to 4.48%. Some individuals showed both rapid and gradual weight loss components.

BODY MASS AND BAT MASS. The average body mass (g) of senescent rats before their spontaneous weight loss (i.e., while they were presenescent) was 430.3 ± 7.8 g, males, and 297.6 ± 8.2 g, females. These values did not differ significantly from those of old rats that remained presenescent during the study: 440.5 ± 7.0 g, males, and 293.5 ± 6.3 g, females. The total mass of interscapular BAT (mg) recovered from senescent rats was 51% lower in males and 62% lower in females compared with their presenescent counterparts (Table 3). When interscapular BAT mass was expressed relative to body mass (mg/g body mass), similar differences in percent mass recovered were seen. There was a main effect of gender that reflected 39% more interscapular BAT (IBAT; mg/g body mass) in presenescent females than in presenescent males. Total BAT mass shown is for the combined interscapular, subscapular, cervical, axillary, and dorsal aortic depots from which the brown adipocytes were isolated. Total BAT mass paralleled IBAT mass.

NE-INDUCED OXYGEN CONSUMPTION. There were no main effects of body weight stability or gender on resting rates of oxygen consumption of brown adipocytes, which averaged as follows: 58 ± 8 (presenescent males), 61 ± 12 (presenescent females), and 58 ± 13 nmol O$_2$·min$^{-1}$·10$^6$ cells$^{-1}$ (presenescent females). With exposure to NE, respiration increased in a concentration-dependent manner in all groups (Fig. 3), but the maximal response ($V_{\text{max}}$) was 48% lower in senescent vs. presenescent males and 51% lower in senescent vs. presenescent females (Table 4). There were significant main effects of body weight stability and gender on sensitivity of cells to NE. The $EC_{50}$ value was lower in senescent vs. presenescent rats and in males vs. females.

CL-316,243 STIMULATION OF OXYGEN CONSUMPTION. The possibility of altered β$_3$-adrenergic components of thermogenesis in rapid senescence was evaluated by exposing brown adipocytes to a single maximal concentration (25 µM) of CL-316,243. Respiration increased severalfold above resting values in all groups after the addition of this agonist, but the maximal response was significantly lower in cells from senescent vs. presenescent rats (56% lower in males and 60% lower in females). Values in the presence of CL-316,243 averaged 763.86 ± 38.36 vs. 323.02 ± 54.28 nmol O$_2$·min$^{-1}$·10$^6$ cells$^{-1}$ in presenescent vs. senescent males, respectively, and 362.64 ± 47.02 vs. 222.85 ± 39.59 nmol O$_2$·min$^{-1}$·10$^6$ cells$^{-1}$ in presenescent vs. senescent females, respectively.

FOOD RESTRICTION, WEIGHT LOSS, AND NE-INDUCED OXYGEN CONSUMPTION. To evaluate the effect of weight loss on oxygen consumption capacity of senescent brown adipocytes, the maximal response to NE was plotted vs. % body weight loss (Fig. 4). There was no relationship in the females ($r^2$ = 0.016). However, in brown adipocytes from senescent males, up to 36.8% of the variation could be accounted for by their weight loss ($r^2$ = 0.368).

To further examine this relationship, 25- to 26-mo-old presenescent male rats were food restricted to 50–60% of ad libitum consumption for 12–28 days, at which point their total percent weight loss ranged from 11.3 to 25.1%. This was comparable to the spontaneous weight loss of the senescent rats. Brown adipocytes isolated from the food-restricted presenescent rats showed con-

<table>
<thead>
<tr>
<th>Table 3. Body and BAT mass in presenescent and senescent F344 rats in experiment 2</th>
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<tr>
<td></td>
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<tr>
<td><strong>Male</strong></td>
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<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>Body mass, g</td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td>Interscapular BAT mass mg</td>
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<tr>
<td>mg/g body mass</td>
</tr>
<tr>
<td>Total BAT mass mg</td>
</tr>
<tr>
<td>mg/g body mass</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. Within a row, means sharing a common letter superscript are not significantly different. Total BAT mass represents combined interscapular, subscapular, cervical, axillary, and dorsal aortic depots from which brown adipocytes were isolated.

Table 4. NE-induced Vmax and EC50 of isolated brown adipocytes from presenescent and senescent F344 rats in experiment 2

<table>
<thead>
<tr>
<th>Vmax, nmol O2·min⁻¹·10⁶ cells⁻¹</th>
<th>EC50, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>Presenescent</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>654 ± 42A</td>
</tr>
<tr>
<td>(12)</td>
<td>(14)</td>
</tr>
<tr>
<td>Female</td>
<td>558 ± 30A</td>
</tr>
<tr>
<td>(14)</td>
<td>(9)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats are shown in parentheses. For each variable, means sharing a common letter superscript are not significantly different. Results are from experiments summarized in Table 2 but are based on evaluation of concentration-response curves for individual rats.
18.6 vs. 66.1 ± 39.5 nmol·h⁻¹·10⁶ cells⁻¹). With exposure to increasing NE concentrations, glycerol release increased in a concentration-dependent manner, with no significant differences in the maximal response of cells from presenescent vs. senescent rats (833.5 ± 133.8 vs. 1,070.5 ± 67.3 nmol·h⁻¹·10⁶ cells⁻¹). Significantly elevated lipolytic activities were also observed in response to maximal (100 µM) forskolin stimulation (Fig. 6B), with no significant differences between presenescent and senescent rats.

Uncoupling protein 1 (UCP-1). The blunted NE- and CL-316,243-induced oxygen consumption of brown adipocytes isolated from senescent rats, in the absence of any deficit in NE-induced cAMP levels or NE-induced lipolysis, led us to hypothesize that the reduced thermogenic response was associated with a reduced cell content of mitochondrial UCP-1. This possibility was evaluated using brown adipocytes isolated from a third group of male F344 rats. The immunoreactive UCP-1 content was indeed significantly less in brown adipocytes isolated from senescent vs. presenescent rats. This was observed when the data were expressed as micrograms per 10⁶ intact cells (72% lower) as well as micrograms per 10⁶ total cells (i.e., intact plus broken cells, 65% lower) (Fig. 7).

**DISCUSSION**

This study was designed to evaluate mechanisms underlying the attenuation of cold-induced thermoregulation and BAT thermogenic capacity observed previously in older male F344 rats (5, 13, 14). Our earlier cross-sectional studies indicated that, as a group, older (23- to 27-mo-old) vs. younger (6- and 12-mo-old) males have significantly lower core temperatures after several hours of cold (5, 13). In addition, we have observed greater cold-induced hypothermia in older males vs. older females (5, 13). The average degree of hypothermia developed by older rats in these investigations was in the range of 1–3°C (5, 13). There is, however, variation in the degree of hypothermia among older rats. Some show no to moderate decreases in core temperature after 4 h at 6°C, whereas others become severely hypothermic (core temperature lower than 32°C). In these earlier studies, we considered older rats exhibiting severe cold-induced hypothermia to be “sick” and excluded them from the analysis (see Ref. 5). However, we have recently found that the development of severe cold-induced hypothermia in older F344 rats is associated with the rapid loss of body weight that occurs near the end of the lifespan (14). Together, these data suggest that there are two components of thermo-
ADRENERGIC RESPONSES OF F344 RAT BROWN ADIPOCYTES

regulatory decline in the aging F344 rat. The first component reflects moderate attenuation that develops over a period of months and that is manifested in the absence of rapid weight loss (for example, in a longitudinal study, we observed that weight-stable older male F344 rats exhibited a 0.9–1.1°C lower cold-induced core temperature at 25.6–26.6 mo of age than at 24 mo (14)]. The second component reflects severe attenuation that develops rapidly in the last few days to weeks of life and that is associated with passage into a different physiological state (i.e., senescence). Rapid body weight loss is a marker, rather than a cause, of this transition from gradual aging to senescence (14). In the present study, experiment 1 focused on 6-, 12-, and 26-mo-old weight-stable rats to evaluate mechanisms underlying the moderate attenuation of cold-induced thermoregulation that is associated with chronological age, whereas the studies on senescent rats (experiment 2) were designed to evaluate mechanisms involved in the severe loss of thermoregulatory ability that is expressed near the end of life.

Experiment 1. Effects of Chronological Age and Gender on Brown Adipocyte Oxygen Consumption

Experiment 1 tested the hypothesis that the attenuated cold-induced BAT thermogenesis (indexed by mitochondrial GDP binding) observed previously in older male vs. younger male and older female F344 rats (13) reflected blunted thermogenesis of brown adipocytes themselves. For this, we measured NE and CL-316,243 (β3-adrenergic agonist) stimulation of oxygen consumption of isolated brown adipocytes and found no major age (6 vs. 12 vs. 26 mo) or gender differences in maximal respiratory responses of brown adipocytes to NE or to CL-316,243. The brown adipocytes from 6- and 12-mo-old males were somewhat more sensitive to NE than were cells from younger females (indicated by the ~2 times lower EC50 values in males vs. females). However, there were no significant differences in sensitivity to NE in brown adipocytes from 26-mo-old males vs. 26-mo-old females. These data indicate that the maximal ability of brown adipocytes to transduce the thermogenic signal via the β3-adrenergic pathway and to generate heat is preserved with advanced chronological age in both males and females. The age/gender differences in cold-induced BAT thermogenesis in vivo may instead involve differences in the number of brown adipocytes (i.e., fewer cells in older males vs. younger males and older females). Alternatively, the brown adipocytes in older males may not respire as well in vivo as they do in vitro, perhaps because of desensitization and/or downregulation of the β3-adrenergic pathway, the presence of inhibitory signals, and/or reduced oxygen availability.

Scarpace et al. (19) showed that the β3-adrenergic agonist CGP-12177A increased the amount of BAT GDP binding in younger but not in older non-cold-exposed male F344 rats. These results suggested that the β3-adrenergic signaling pathway for BAT thermogenesis was impaired or absent with aging in males. However, our data indicate that brown adipocytes from 26-mo-old male rats are capable of β3-mediated thermogenesis in vitro when maintained at 37°C in an oxygenated Krebs-Ringer bicarbonate buffer media. Because CL-316,243 is highly selective for β3-adrenergic receptors (1), it is unlikely that the CL-316,243-induced increase in oxygen consumption rate of isolated brown adipocytes was mediated through β1- or β2-receptors. NE binds to all β-receptor subtypes, but the fact that

Fig. 6. Glycerol release from brown adipocytes incubated in KRBA in the absence or presence of various NE concentrations (A) or maximal (100 µM) forskolin (B). Results are means ± SE for presenescent (n = 8) and senescent (n = 5) male F344 rats. Curves in A were drawn as described for NE in Fig. 1, where glycerol release is expressed as increase over resting level. Bars sharing a common letter do not differ significantly (P > 0.05).

Fig. 7. Immunoreactive uncoupling protein 1 (UCP-1) content of isolated brown adipocytes from presenescent (n = 8) and senescent (n = 13) male F344 rats. Proteins from 10.7 µg of each cell suspension were separated by SDS-PAGE and detected by immunoblotting as described in MATERIALS AND METHODS. Intact values were calculated on basis of no. of intact cells only; total represents values calculated on basis of no. of intact plus broken cells. *P < 0.05, significantly lower UCP-1 content of senescent vs. presenescent cells.
maximal NE- and CL-316,243-induced oxygen consumption rates were comparable suggests that the amount of cAMP generated via the β_3-adrenergic pathway alone is sufficient to induce the maximal thermogenic response. At present, it is unclear what mechanism(s) prevents β_3-mediated BAT thermogenesis in older male rats in vivo, or, alternatively, what enables β_3-mediated thermogenesis of "old" brown adipocytes in vitro. The difference may reflect differences in the environment of cells (e.g., temperature, pH, inhibitory signals). Nonetheless, it is apparent that in both males and females there are at least some brown adipocytes that retain thermogenic responsiveness in old age. Future studies in this area should include measurement of BAT cellularity (to evaluate whether the age/gender differences in cold-induced BAT thermogenesis reflect differences in the number of brown adipocytes) and exploration of the mechanisms responsible for the in vivo vs. in vitro differences in β_3-adrenergically mediated responses of BAT depots vs. isolated brown adipocytes.

**Experiment 2. Effect of Senescence on Brown Adipocyte Oxygen Consumption, cAMP, Lipolysis, and UCP-1.**

We previously reported that cold-induced thermoregulation and BAT thermogenic capacity (measured by the amount of UCP-1) deteriorate dramatically as older male F344 rats approach the end of their life span (14). This transition from gradual aging, i.e., chronological aging, to the end-of-life physiological state, a state that we refer to as senescence, occurs spontaneously and is marked by reduced food intake, which causes rapid weight loss. The experiments in the second part of this investigation tested the hypothesis that senescence decreased the thermogenic capacity of individual brown adipocytes. Our data support this hypothesis. We found that isolated brown adipocytes from senescent rats (22- to 26-mo-old rats with rapid weight loss) consumed 51–60% less oxygen during maximal stimulation with NE or CL-316,243 compared with cells from presenescent rats (26-mo-old rats with a stable body weight). This occurred in males as well as in females, with no gender effect. This attenuation of maximal agonist-induced BAT thermogenesis reflect differences in the number of brown adipocytes) and exploration of the mechanisms responsible for the in vivo vs. in vitro differences in β_3-adrenergically mediated responses of BAT depots vs. isolated brown adipocytes.

Although the blunted adrenergically induced oxygen consumption of senescent brown adipocytes cannot be explained by reduced cAMP levels or lipolysis, the same cannot be said for the brown adipocyte-specific UCP-1. Our data demonstrate that UCP-1 levels are significantly lower (65–72%) in senescent vs. presenescent brown adipocytes (Fig. 7). This loss of UCP-1 could account for the 51–60% reduction in maximal NE- and CL-316,243-induced oxygen consumption. Nevertheless, it is premature to conclude from the UCP-1 data alone that its reduction is the only dysfunction in senescent brown adipocytes. For example, there may be a reduced number of mitochondria per cell and/or altered concentrations/activities of respiratory chain components. Also, the functional activity of the immunoreactive UCP-1 molecules may be compromised in senesence.

BAT UCP-1 levels vary with the metabolic requirements of the animal, provided that the necessary regulatory signals and pathways are intact and functional. Factors known to have important influences on UCP-1 levels include NE (10), thyroid hormone (20), and insulin (6). Our preliminary data indicate that senescent rats have significantly lower concentrations of serum total thyroxine and free thyroxine (1.74 ± 0.09 µg/dl; 0.14 ± 0.00 ng/dl) than do presenescent rats (3.20 ± 0.87 µg/dl; 0.43 ± 0.06 ng/dl). Thus reduced levels of this hormone signal to BAT may contribute to the lower UCP-1 levels that occur with senescence. Blunted sympathetic (NE) signaling may also occur, a possibility that we are currently evaluating.

It is unclear what physiological stimulus triggers the onset of senescence and what mechanisms are responsible for the rapid deterioration of the thermoregulatory system that is associated with it. However, it does not appear to be due simply to a reduction in food intake and the concomitant loss of body weight. As illustrated in Fig. 4, there was no decrease in maximal NE-induced oxygen consumption of brown adipocytes from 25- to 26-mo-old presenescent male rats that were food restricted to lower their body weight by the same amount as that of the senescent rats. This is consistent with results from our previous study, in which we observed no detrimental effect of weight loss from food restriction on cold-induced thermoregulation of 25- to 26-mo-old presenescent male F344 rats (14). These
findings have led us to conclude that rapid body weight loss is a marker, rather than a cause, of the physiological alterations manifested at the level of the whole animal and intact brown adipocytes near the end of the life span of F344 rats and that other factors associated with the transition from gradual aging to senescence are responsible.

The transition from gradual aging to senescence may reflect dysfunction of the hypothalamus. Support for this hypothesis derives from the observation that food intake and cold-induced thermoregulation, two major systems that are regulated by the hypothalamus, begin to deteriorate at approximately the same time (14). Hypothalamic dysfunction could also account for the reduced UCP-1 levels measured in BAT depots (14) and in isolated brown adipocytes from senescent rats if there were functional alterations in neurons that regulate sympathetic activity to BAT. That is, BAT UCP-1 levels, which are highly regulated by the sympathetic nervous system, may not be maintained as well in senescent vs. presenescent rats because of blunted sympathetic signaling, which in turn reflects altered hypothalamic function. If alterations in central nervous system function do occur, it remains to be determined whether this reflects pathology or a nondisease biological phenomenon (i.e., some regulated event leading to the rapid demise of the aged animal).

The diseases and lesions most common in aged F344 rats include granular cell leukemia, glomerulonephropathy, interstitial cell tumors of the testes, and pituitary cysts/adenomas (9). The effect that these pathologies may have had on parameters measured in the present investigation is not known, but those interfering with hypothalamic function. If alterations in central nervous system function do occur, it remains to be determined whether this reflects pathology or a nondisease biological phenomenon (i.e., some regulated event leading to the rapid demise of the aged animal).

The diseases and lesions most common in aged F344 rats include granular cell leukemia, glomerulonephropathy, interstitial cell tumors of the testes, and pituitary cysts/adenomas (9). The effect that these pathologies may have had on parameters measured in the present investigation is not known, but those interfering with energy balance would be of particular concern. Negative energy balance, manifested by weight loss, can result from a reduction in energy intake (decreased food consumption and/or impaired nutrient absorption) and/or inappropriately high rates of energy expenditure. In the rat, cachexia (body wasting) experimentally induced by T-cell leukemia is associated with both hypophagia and hypermetabolism, the latter being due, at least in part, to elevated BAT thermogenesis (indexed by mitochondrial GDP binding (18)). Elevated BAT thermogenesis has also been reported in rats bearing solid tumors (17). Our previous data suggest that senescence is not simply a reflection of cachexia, because there was no increase in resting whole animal metabolic rate and no depletion of body protein in senescent F344 rats with 6–15% weight loss (14). It is also worth noting that, whereas increased BAT thermogenesis appears to be a contributing factor in the weight loss associated with cancer-related cachexia, our current and previous data are consistent with reduced, rather than enhanced, BAT thermogenesis in senescence. A second pathological concern in relation to energy balance and senescence is the presence of pituitary cysts that, when large enough, can deform or compress the hypothalamus and possibly affect energy balance in this indirect way. Approximately 40–50% of the senescent rats in the present study had pituitary cysts, but in only a few of these rats were they large enough to alter the appearance of the hypothalamus. There was no correlation between the presence and/or size of these cysts and the maximal NE-induced thermogenesis of senescent brown adipocytes. Thus it is unlikely that this pathology underlies the transition from aging to senescence or the changes in brown adipocyte metabolism that are associated with it.

Conclusions

We have shown that maximal NE- and CL-316,243 (β3-adrenergic)-induced oxygen consumption (thermogenesis) of isolated brown adipocytes does not diminish with advanced chronological age in males or females (6- vs. 12- vs. 26-mo-old rats with a stable body weight) but is greatly attenuated in older (22–26-mo-old) male and female F344 rats exhibiting a rapid loss of body weight as occurs in the last few days to weeks of life (14). This attenuation of adrenergically mediated thermogenesis with senescence was associated with lower UCP-1 concentrations but no alterations in NE-induced cAMP levels or lipolysis of brown adipocytes. These data indicate that, in both males and females, senescence, but not chronological age, significantly impacts NE-β3-mediated thermogenesis of brown adipocytes in vitro and that this effect involves altered mitochondrial rather than altered membrane or cytosolic events. Evaluation of factors that underlie this function loss with senescence is currently in progress.

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