Influence of Age and Gender on Brown Adipose Tissue Norepinephrine Turnover

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Abstract. **We hypothesized that the attenuated brown adipose tissue thermogenic capacity observed previously in cold-exposed 27-month-old male versus female Fischer 344 rats might result, in part, from blunted sympathetic signaling to the tissue. As an index of sympathetic activity to brown fat, norepinephrine (NE) turnover in this tissue was evaluated at rest (22–24°C) and during 1.5 hr of cold exposure (6°C) in male and female Fischer 344 rats, aged 6, 12, and 26 months. Resting NE turnover as well as the rate constant for NE efflux from brown fat, expressed as total and as per gram of tissue protein, did not, in general, differ from age or gender. During cold exposure, rate constants and NE turnover rates increased significantly from those at rest in all groups. Brown fat NE turnover in cold-exposed 26-month-old male rats was greater than that observed in age-matched females, suggesting greater, not less, sympathetic signaling in the males versus females. These data indicate that the attenuated brown fat thermogenic capacity as well as the blunted cold-induced thermogenic responses of cold-exposed older male versus female rats reported previously cannot be explained by diminished release of NE from sympathetic nerves innervating brown adipose tissue.**

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We reported previously that brown adipose tissue thermogenic capacity of cold-exposed (6 hr at 6°C) female Fischer 344 (F344) rats, ages 5, 23, and 27 months, was significantly greater than that of age-matched males (1). Females retained brown fat mass and mitochondrial binding of guanosine-5′-diphosphate (GDP) with advancing age, while males showed declining mass and GDP binding. These findings suggest that a major component of the changes in thermoregulatory responses to cold with age is gender dependent in that 27-month-old female rats have greater brown adipose tissue (BAT) nonshivering thermogenic capacity than do their male counterparts.

Because brown fat thermogenesis is regulated to a large degree by the level of sympathetic stimulation to brown fat cells (2, 3), we postulated that the observed gender-related differences in brown fat thermogenic capacity might result in part from altered sympathetic activity to the tissue. This suggestion is consistent with the data of McCarty (4), who reported significantly lower concentrations of plasma norepinephrine (NE) after the acute stress of foot shock in 24- versus 4-month-old male F344 rats. McCarty (4) concluded that impaired central stimulation of efferent sympathetic pathways in the 24-month-old rat could explain the differences in plasma NE concentrations.

The purpose of this investigation was to evaluate the possibility that age and gender result in altered sympathetic stimulation of brown fat. Specifically, we tested the hypothesis that the attenuated brown fat thermogenic capacity and cold-induced responsiveness of cold-exposed 26-month-old male versus female F344 rats reflect blunted sympathetic signaling to the tissue by measuring brown fat NE turnover.

Materials and Methods

Animals and Animal Care. Male inbred F344 rats ages 6, 12, and 26 months were obtained from the National Institute of Aging animal colony maintained by Harlan Sprague-Dawley Laboratory (Indianapolis, IN). On arrival, the rats were placed in a laminar flow

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unit (Duo-Flo; Lab Products, Maywood, NJ) that provided clean air by precirculation through high-efficiency particle filters. Rats were individually housed in wire-bottom hanging cages (20 × 25 × 18 cm) and maintained on a 12:12-hr light:dark cycle (lights on at 0600 hr, off at 1800 hr) at a room temperature of 25–26°C. Rats were allowed *ad libitum* access to food (autoclaved NIH-31 chow; Tekland Research Diets, Indianapolis, IN) and water (distilled and acidified to pH 3.5). A detailed description of the procedures for maintenance of this colony is reported elsewhere (5). All rats were maintained in our facility for 2 weeks before experimentation.

Rats were examined daily for signs of external disease; no rats had overt signs of disease. At the time of death, a systematic, visual necropsy was performed on all rats. Many of the 26-month-old rats had testicular tumors, and some small tumors were present in other areas (pathologies were not obtained on any of these tumors). None of these rats were excluded from the study.

Norepinephrine Turnover. Norepinephrine turnover in brown fat of 10- to 12-hr fasted male and female rats was measured for 7 hr at room temperature (22–24°C) and during 1.5 hr of cold exposure (6°C) using the method of competitive inhibition of tyrosine hydroxylase by α-methyl-*p*-tyrosine (α-MPT) as described by Brodie *et al.* (6). Pilot investigations in rats indicated that interperitoneal injection of α-MPT versus volume-matched saline during cold exposure resulted in significantly greater decreases in body temperature of all age/gender groups by 1.5 hr. Thus, we selected 1.5 hr of cold exposure in order to limit the effects that α-MPT may have on systemic thermoregulation. For each age and gender, five time/temperature groups of five to seven animals/group were used. Group 1 animals were sacrificed immediately after interperitoneal injection of saline and were used to establish initial tissue concentrations of NE for both the cold-exposed and room-temperature (rest) treatments. Groups 2 and 3 animals were injected interperitoneally with 250 mg/kg of α-MPT and were then exposed to 45 and 90 min of 6°C ambient temperature, respectively. Groups 4 and 5 animals also received 250 mg/kg of α-MPT at Time 0. Rats in Group 4 were sacrificed after exposure to 22–24°C for 3 hr. Group 5 rats received an additional injection of α-MPT, 125 mg/kg, at 3.5 hr of 22–24°C and were then sacrificed 3.5 hr later (i.e., total exposure at room temperature = 7 hr).

Analysis of Brown Fat Norepinephrine Content. Rats were sacrificed by decapitation, and the interscapular brown adipose tissue was rapidly removed, blotted dry, weighed, quickly frozen in liquid N_2, and stored at -70°C. The frozen tissues were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) in 10 vol (v/w) of a solution (pH 2.6) containing 0.12 *M* acetic acid, 0.3 m*M* sodium bisulfite, 0.4 m*M* EDTA, and 3,4-dihydroxy benzylamine (Sigma Chemical Co., St. Louis, MO) at known concentrations of 250–350 fmol/μl. The latter was used as the internal standard for high-performance liquid chromatography.

The brown fat homogenates were sonicated on ice three to five times for 5 sec and then centrifuged for 20 min at 13,000×*g*. Approximately 250 μl of the non-lipid-containing supernatant was removed and passed through a 0.45-μm filter. Forty microliters of this filtrate were injected into the high-performance liquid chromatography, and NE was separated on a reverse phase column (Spherisorb OdS2, 5 μm, 15 cm × 4.6 mm) at a flow rate of 1.0 $ml \cdot min^{-1}$. The mobile phase contained 70.0 m*M* citric acid, 34.5 m*M* NaOH, 0.31 m*M* 1-octanesulfonic acid, 0.30 m*M* Na_2 EDTA, and 5% acetylnitrile (pH 2.9). The concentration of NE in the sample was determined by electrochemical detection at an applied potential of 0.62 V. The chromatograms were analyzed using Baseline 810 Chromatography Workstation Software (Dynamic Solution, Ventura, CA).

Statistics. Analysis of variance with a factorial design (age and gender as the main effects) was used to evaluate the data. When a significant effect was found, Fisher's least significant difference post hoc test was used to evaluate differences between the groups. Differences were considered significant at $P < 0.05$. Statistical analysis of NE turnover was performed according to the method described by Taubin *et al.* (7)

Results

Body and Brown Fat Weight. As expected, there was a significant effect of age and gender on body weight (Table I). That is, male rats weighed more than females, and 12-month-old rats weighed more than did 6-month-old rats. The fact that male 26-month-old rats weighed significantly less than did the 12-month-old rats resulted in a significant interaction of age and gender.

Interscapular brown fat weight (mg) was significantly greater in male versus female 6-month-old rats, but not at any other age. However, when the brown fat data were expressed as $mg \cdot g$ body wt^{-1}, female rats had significantly more than did their age-matched male counterparts. There was also a significant effect of gender on brown fat protein content when the data were expressed as either total mg or $mg \cdot g$ brown fat^{-1}, with values in females being consistently higher than those in males.

Colonic Temperatures. Colonic temperature at Time 0 did not differ among the gender and age groups (Table II). As expected from our pilot data, 90 min of exposure to 6°C resulted in declining colonic temperature in all gender/age groups, although the decline was not significant in the 6-month-old female rats.

Table I. Body Weight and Interscapular BAT Weight and Interscapular BAT Protein in Male and Female F344 Rats at Time 0[a]

Age	Body wt (g)	Interscapular BAT wt		Interscapular BAT protein	
		(mg)	(mg/g body wt)	(mg)	(mg/g BAT wt)
6 Months					
Female	207.2 ± 4.3*	171.9 ± 13.8*	0.83 ± 0.06*	41.57 ± 4.90*†	242.79 ± 21.91*
Male	344.5 ± 16.1‡	234.8 ± 13.0‡	0.68 ± 0.05‡	37.80 ± 2.78*	162.05 ± 11.72‡
12 Months					
Female	261.8 ± 8.1†	251.5 ± 13.3‡	0.96 ± 0.04*	56.5 ± 9.39‡	227.07 ± 41.67*
Male	451.9 ± 12.5§	247.5 ± 14.8‡	0.55 ± 0.04‡	36.34 ± 2.80*	147.39 ± 9.43‡
26 Months					
Female	288.4 ± 12.1†	261.0 ± 38.1‡	0.89 ± 0.10*	50.77 ± 7.8†‡	203.34 ± 35.6*
Male	385.6 ± 11.9‡	239.1 ± 28.5‡	0.61 ± 0.06‡	38.24 ± 5.45*	159.4 ± 7.05‡

[a] Values are means ± SE; n = 5–6 per group. Within a column, values sharing a common symbol (*, †, ‡, §) are not significantly different ($P < 0.05$).

Table II. Colonic Temperature of Female and Male Rats During Norepinephrine Turnover Experiment

	Cold exposure		
	0 min	45 min	90 min
6 Months			
Female	37.1 ± 0.2	37.1 ± 0.4^{a}	36.3 ± 0.2
Male	36.8 ± 0.9^{A}	35.8 ± 1.0^{bcB}	35.7 ± 0.2^{B}
12 Months			
Female	37.3 ± 0.3^{A}	35.7 ± 0.4^{bcB}	36.1 ± 0.5^{B}
Male	37.0 ± 0.3^{A}	35.5 ± 0.4^{cB}	35.9 ± 0.5^{B}
26 Months			
Female	37.0 ± 0.3^{A}	36.0 ± 0.3^{abcAB}	35.3 ± 0.6^{B}
Male	36.7 ± 0.1^{A}	36.7 ± 0.2^{abA}	34.8 ± 0.7^{B}

Note. Values are mean ± SE; n = 5–6 animals per group. Within a column, values sharing a common lower case letter are not significantly different ($P < 0.05$). Within a row, values sharing an upper case letter are not significantly different ($P < 0.05$).

Norepinephrine Turnover. Although some differences in initial steady state tissue concentrations of NE were observed, no consistent pattern was noted (Tables III and IV). Resting NE turnover as well as the rate constant for NE efflux from brown fat, expressed as total or per gram of brown fat protein, were significantly greater in 6-month-old female versus male rats. No other differences were observed at rest. During cold exposure, rate constants for NE efflux and NE turnover rates increased significantly from those at rest in all groups. When the data were expressed in terms of total interscapular brown fat (Table IV) or per gram of brown fat protein (Table III), the NE rate constant and turnover rates were significantly greater in 26-month-old male versus female rats. In contrast, the rate of NE turnover in 6-month-old female rats, expressed as $nmol \cdot hr^{-1}$/interscapular brown fat pad, was greater than that for similarly aged male rats.

Discussion

Activation of brown fat thermogenesis is mediated primarily by norepinephrine released from sympathetic innervation to the adipocyte (2), as is activation of the expression of uncoupling protein, an index of the thermogenic capacity of brown adipocytes (3). Our previous findings that older male rats are less responsive to norepinephrine than are younger animals (5) and that 26-month-old males exhibit lower brown fat mass, protein, and cold-induced mitochondrial GDP binding (a measure of functional uncoupling protein) than do 26-month-old females (1) led us to hypothesize that these changes may reflect blunted sympathetic signaling in older males. The data in the present study negate this hypothesis. That is, we found that the rate of NE release from brown fat of cold-exposed 26-month-old males was in fact higher, not lower, than in their female counterparts, suggesting more, rather than less, sympathetic activity in response to cold. Furthermore, NE content and turnover at rest did not diminish in the older rat, implying that factors other than decreased sympathetic signaling are responsible for the reduced thermogenic potential of the older males.

The mechanism(s) underlying the increased release of NE by sympathetic nerves innervating BAT of the older male rat is unclear. It is possible that the enhanced sympathetic activity of BAT observed in the male rat is a compensatory response to this tissue's attenuated thermogenic and/or metabolic capacity (1). In contrast, because brown fat thermogenic capacity in older females does not decrease with age as much as in the male, sympathetic signaling during cold exposure does need to increase to the same level as in the male. The concept that one component of the sympathetic pathway compensates for declining activity/capacity of another is consistent with the work of Mazzeo and Grantham (8). These investigators observed increased NE turnover and NE rate constants in hearts of 25- versus 6-month-exercised female F344 rats. Because several previous investigations have documented an age-related attenuation in adrenergic receptor responsiveness of the heart, Mazzeo and Grantham (8) concluded that the

Table III. Norepinephrine Content, Rate Constant, and Turnover in Interscapular BAT, Expressed per mg of Interscapular BAT Protein, During Rest and Cold Exposure (6°C)[a]

Age	$[NE_0]$ pmol·mg prot^{-1}	Rate constant hr^{-1}		Turnover pmol·hr^{-1}·mg prot^{-1}	
	Rest	Rest	Cold	Rest	Cold
6 Months					
Female	78.94 ± 6.65*	0.24 ± 0.02[b]	0.62 ± 0.09	19.10 ± 3.15*	49.19 ± 11.35*,†
Male	71.68 ± 7.67*,†	0.13 ± 0.03	0.41 ± 0.13	9.52 ± 3.1†	29.22 ± 24.90†,‡
12 Months					
Female	42.99 ± 3.93†	0.13 ± 0.03	0.46 ± 0.12	5.40 ± 1.84†	19.95 ± 7.07‡
Male	60.21 ± 2.65†	0.10 ± 0.02	0.47 ± 0.08	5.96 ± 1.42†	28.53 ± 12.44‡
26 Months					
Female	52.39 ± 14.50†,‡	0.24 ± 0.05	0.48 ± 0.19[b]	12.46 ± 5.96*,†	24.94 ± 17.01†,‡
Male	68.65 ± 7.00*,†	0.26 ± 0.03	0.86 ± 0.10	17.57 ± 4.09*	59.30 ± 13.20*

[a] Values for norepinephrine concentration $[NE_0]$ and rate constant are means ± SE. Values for turnover are means ± ½ the 95% confidence interval (7). Within the columns of $[NE_0]$ and rest and cold NE turnover, values sharing a common symbol (*, †, ‡) are not significantly different ($P < 0.05$).
[b] By *t* test, value is significantly different from male rat of the same age.

Table IV. Norepinephrine Content, Rate Constant, and Turnover in Interscapular BAT Pads During Rest and Cold Exposure (6°C)[a]

Age	$[NE_0]$ nmol	Rate constant hr^{-1}		Turnover nmol·hr^{-1}	
	Rest	Rest	Cold	Rest	Cold
6 Months					
Female	3.20 ± 0.37*	0.22 ± 0.02[b]	0.54 ± 0.08	0.72 ± 0.16*	1.73 ± 0.45*
Male	2.60 ± 0.08*,†	0.08 ± 0.02	0.38 ± 0.12	0.23 ± 0.06†	0.98 ± 0.31†
12 Months					
Female	2.44 ± 0.37†	0.13 ± 0.05	0.65 ± 0.17	0.32 ± 0.17†	1.58 ± 0.64*
Male	2.17 ± 0.18†	0.08 ± 0.02	0.32 ± 0.10	0.18 ± 0.06†	0.69 ± 0.27†
26 Months					
Female	2.22 ± 0.18†	0.21 ± 0.03	0.39 ± 0.17[b]	0.48 ± 0.11*,†	0.86 ± 0.47†
Male	2.45 ± 0.15*,†	0.23 ± 0.03	0.77 ± 0.07	0.57 ± 0.11*	1.89 ± 0.31*

[a] Values for norepinephrine concentration $[NE_0]$ and rate constant are means ± SE. Values for turnover are means ± ½ the 95% confidence interval (7). Within the columns of $[NE_0]$ and rest and cold NE turnover, values sharing a common symbol (*, †) are not significantly different ($P < 0.05$).
[b] By *t* test, value is significantly different from male rat of the same age.

appropriate exercise heart rate in aging animals is maintained through greater sympathetic stimulation. In any case, the data of Mazzeo and Grantham (8) and those presented here indicate that elements of the efferent sympathetic nervous system pathway are intact and functional in the aged male and female F344 rat, and they support the idea that neural stimulation may increase in response to a decrease in end-organ responsiveness.

Heat generation by the brown adipocyte occurs via a complex set of reactions involving β-adrenergic receptor-stimulated adenylate cyclase activity and culminating with activation of a uniquely expressed protein (uncoupling protein) that short-circuits the proton-motive force across the inner mitochondrial membrane. One possible site of the age/gender differences in brown fat thermogenic capacity may involve adrenergic reception and/or signal transduction. Scarpace *et al.* (9) reported that β-receptor density as well as tissue-specific isoproterenol stimulation of adenylate cyclase is 40–50% lower in brown fat membranes isolated from 24- versus 3-month-old F344 male rats. Furthermore, attenuated receptor-stimulated adenylate cyclase activity has been reported in parotid and submandibular salivary glands (10) and in renal membranes isolated from old versus young rats (11). Although the effect of age and/or gender on possible alterations in postreceptor signal transduction in brown fat has yet to be elucidated, we have found that blunted NE-induced BAT thermogenesis of older male rats is correlated with the availability of GDP-binding sites (i.e., cold-induced GDP binding) rather than altered NE sensitivity (5).

The identification of a mechanism leading to an age-related loss in cellular homeostasis and, thus, cellular senescence has not yet been elucidated. Although many investigations have identified alterations to specific regulatory mechanisms in various cell types that may partially explain aging, it is more likely that cellular

aging reflects the interactions among many levels of regulation (i.e., metabolic, hormonal, gene expression, etc.). The fact that (i) brown fat thermogenesis declines in aging male rats, (ii) we know the major metabolic pathways regulating thermogenesis in brown fat, and (iii) these regulatory mechanisms are directly coupled to the expression of a unique protein required for thermogenesis makes brown fat an ideal model for studying the regulation of cellular aging. That is, investigations evaluating alterations in the regulation of brown fat thermogenesis may provide a model by which we can gain insight into mechanisms leading to declining cellular homeostasis during senescence.

The data presented here suggest that the lower brown fat thermogenic capacity and thermogenic responsiveness of older male versus female rats are not due to attenuated sympathetic signaling to BAT, as indicated by rates of NE release from BAT. Rather, the differences in brown fat thermogenic capacity and responsiveness in male versus female rats may reflect alterations in the adipocyte with respect to signal transduction and/or metabolic pathways.

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