The Serum Levels of 17 β -estradiol, Progesterone and Triiodothyronine Correlate with Brown Adipose Tissue Thermogenic Parameters During Aging

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Key Words

Sex steroids • Thyroid hormones • T3 • UCP1 • Mitochondria

Abstract

Sex and thyroid hormones are among the factors modulating energy metabolism through regulation of mitochondrial oxidative capacity. Brown adipose tissue (BAT) in old female rats has been shown to maintain, better than males, the ability to produce heat when exposed to cold. Considering the decline that takes place in gonadal and thyroid function during aging, the aim of this work was to test whether the age-related hormonal status may be a potential mediator of the gender differences in BAT decline. COX activity, UCP1, mitochondrial respiration rate, mitochondrial DNA (mtDNA), levels of mitochondrial transcription factor A (TFAM), as well as the serum levels of sex steroids (17 β -estradiol, progesterone and testosterone) and thyroid hormones (T3 and T4) were measured in 6-, 18-, and 24-month (mo) old male and female rats kept at 22 °C. Six mo-old female rats showed higher thermogenic features than males. Male rats at 18-mo showed a decrease in uncoupling activity compared to females. Both genders showed marked signs of atrophy in BAT of 24-mo-old rats,

characterized by a decrease in total DNA, mitochondrial protein, COX and UCP1, whereas mtDNA was found to increase. Sex steroid hormones were well correlated with BAT parameters when both genders were considered, however, T3 was the hormone with the strongest positive correlations in female rats. In conclusion, our findings provide evidence suggesting that T3 may be a potential mediator of the sexual dimorphism in the effect of aging on the functional decline of BAT.

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Introduction

The ability to regulate body temperature diminishes with age both in human and rodents [1, 2]. These alterations often result in a decrease in cold tolerance and in a lower control of body weight, contributing to the age-related increase in adiposity. In rodent models, this decline in thermoregulation has been related, among other factors, to attenuated brown adipose tissue (BAT) heat production [3]. BAT is a tissue specialized in releasing energy as heat through the action of its characteristic uncoupling protein 1 (UCP1), an inner membrane mito-

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chondrial protein able to short-circuit the proton gradient generated by the respiratory chain.

The age-related decline in BAT thermogenesis has been shown to be strongly influenced by gender [4]. Old female rats are able to maintain a higher ability to respond to cold exposure, showing a greater BAT mass, protein and uncoupling protein 1 (UCP1) content and activity (measured as GDP binding) than their similar-aged male rats [5]. These age and gender differences in uncoupling capacity are abolished when these variables are expressed independently of mitochondrial or total protein [2, 6], suggesting that these differences rely on changes in the amount of mitochondria and/or functional BAT. However, the effect of age and gender on BAT mitochondrial biogenesis has not been explored yet.

Mitochondrial biogenesis is a highly complex process that includes both mitochondrial proliferation and differentiation which needs the coordination of both nuclear and mitochondrial genome. The discovery of mitochondrial transcription factor A (TFAM), which is basic to initiation of both replication and expression of mitochondrial DNA (mtDNA) [7, 8] has opened a new perspective for the study of regulation and coordination of the thermogenic response in the brown adipocyte.

On the other hand, although norepinephrine (NE) is a major regulator of BAT thermogenesis, it has been demonstrated that the blunted thermogenesis of old male versus female rats cannot be explained by diminished sympathetic activity [9]. Neither has age-related deficit in the NE responsiveness of isolated brown adipocytes been found, which suggests that other factors rather than altered NE/ β_3 -AR signalling may be responsible for the BAT functional decline [10].

Apart from NE, production of heat can also be influenced by sex steroids and thyroid hormones [11, 12]. Sex steroids have been shown to modulate the expression of the different adrenoceptor subtypes in primary cultures of brown adipocytes [13] as well as the expression of mitochondrial proteins [14], suggesting a role for sex steroid hormones on the modulation of thermogenic capacity and activity. Particularly, experiments on ovariectomized animals have demonstrated that estrogens play an important role regulating energy expenditure through the activity of UCP1 in BAT [15, 16]. Similarly, thyroid hormones by means of their active form (3,5,3'-triiodothyronine, T₃), are able to modulate the sympathetic stimulation of UCP1 transcription as well as mitochondrial biogenetic processes [17, 18]. In turn, sex hormones exert influence on thyroid function [19, 20], and it is well known that both systems are altered with age [21].

Taking this into account, the aim of this work was to test whether the age-related hormonal status may be a potential mediator of the gender differences in BAT decline. To tackle this aim we characterize in detail the BAT decline in thermogenic capacity by measuring parameters such as BAT composition, UCP1 content, mitochondrial respiration rate and COX activity, as well as the changes in mitochondrial biogenesis (mtDNA, TFAM) in relation to steroid and thyroid status in 6-, 18- and 24-mo-old male and female rats.

Materials and Methods

Animals

All animals were treated in accordance with the university bioethical committee guidelines for animal care and EU regulations (86/609/EEC). Male and Female Wistar rats were purchased from Charles River (Barcelona, Spain) and housed in cages in a temperature controlled room (22 °C) with 12-h light-dark cycle. Rats were fed ad libitum with standard chow pellets (A04, Panlab). The animals were sacrificed at 6, 18 and 24 months of age (n=6-8 per group). Before sacrifice all animals were visually inspected. No pathological signals were found, enough to warrant exclusion of any of the old rats.

Sacrifice and isolation of BAT mitochondria

Animals were killed by decapitation at the start of the light phase. White adipose depots (inguinal, gonadal, retroperitoneal and mesenteric) were dissected and weighed. Brown adipose tissue (BAT) was removed from the interscapular region and carefully dissected in order to prevent any contaminating muscle, connective or white adipose tissue. The tissue was weighed and homogenized in isolation buffer (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.2, 4 °C) in a Teflon/glass homogenizer. The homogenate was filtered through a layer of gauze. Aliquots were used for determination of total protein and triglyceride content as described elsewhere [22, 23] and also cytochrome c activity and western blotting measurements (see below). The rest of the homogenate was used for isolation of mitochondria by differential centrifugation. The nuclei and cell debris were removed by centrifugation at 500xg for 10 min. The supernatant was centrifuged at 8000xg to yield the mitochondrial pellet. The pellet was washed once by resuspension, centrifuged (8000xg) and the final pellet was resuspended in the same buffer. The gravitational force used was chosen in order to achieve a representative fraction of whole mitochondrial population preserving integrity [24]. Mitochondrial protein was measured by the Bradford method [22].

Mitochondrial respiration rate

Mitochondria were incubated in a water-thermostatically regulated chamber with a computer-controlled Clark-type O₂ electrode (Oxygraph; Hansatech, Norfolk, UK) at a concentration of 0.15 mg mitochondrial protein/mL in buffer containing

	6 mo	Male		6 mo	Female		ANOVA
		18 mo	24 mo		18 mo	24 mo	
Body weight (g)	483 ±13	579 ±16	687 ±37	332 ±7	352 ±14	395 ±16	Aabc, G
WAT weight (g)	30.8 ±3.3	59.8 ±5.0 ^a	85.7 ±9.5 ^{bc}	12.9 ±2.0 ^g	27.7 ±2.3 ^{ag}	38.3 ±4.9 ^{bcg}	Aabc, G, AxG
mg/ g body weight	61.1 ±5.8	103 ±1	124 ±1	48.9 ±6.8	77.7 ±4.1	95.9 ±8.7	Aabc, G
BAT weight (g)	0.550 ±0.027	1.16 ±0.12 ^a	1.05 ±0.14 ^b	0.327 ±0.019 ^g	0.577 ±0.039 ^g	0.766 ±0.076 ^{bc}	Aabc, G, AxG
mg/ g body weight	1.14 ±0.09	2.01 ±0.20 ^a	1.51 ±0.16 ^c	1.62 ±0.08 ^g	1.64 ±0.08	1.92 ±0.13 ^{cg}	AxG

Table 1. Effect of age and gender on body, WAT and BAT weight. WAT weight was considered as the sum of inguinal, retroperitoneal, mesenteric and gonadal white adipose tissue depots. Data are shown as mean ± SEM of 6-8 animals. Two-way ANOVA ($p < 0.05$): A, effect of age; G, effect of gender; AxG interaction of age and gender; NS, no significant difference. Post hoc analysis of age (LSD test; $p < 0.05$): a= 6 vs 18 mo, b= 6 vs 24 mo, c= 18 vs 24 mo. Student's t-test ($p < 0.05$): g = female vs. male.

	6 mo	Male		6 mo	Female		ANOVA
		18 mo	24 mo		18 mo	24 mo	
DNA							
mg/g tissue	2.47 ±0.09	2.06 ±0.32	1.29 ±0.25	3.67 ±0.23	2.86 ±0.55	1.06 ±0.10	Ab
mg/depot	1.63 ±0.05	2.04 ±0.31	1.42 ±0.43	1.71 ±0.14	1.72 ±0.35	0.87 ±0.06	Ac
Triglyceride							
mg/g tissue	217 ±26	234 ±29	305 ±48	284 ±35 ^a	324 ±52 ^g	272 ±25 ^c	G, AxG
mg/mg DNA	86.9 ±8.3	171 ±28	246 ±18	79.0 ±9.7	144 ±26	258 ±31	Aabc
Protein							
mg/g tissue	47.8 ±3.0	24.2 ±1.5 ^a	15.8 ±1.6 ^b	77.6 ±3.7 ^g	31.7 ±2.4 ^{ag}	16.3 ±2.0 ^{bc}	Aabc, G, AxG
mg/mg DNA	20.1 ±1.2	10.1 ±1.1	15.1 ±1.1	22.2 ±1.4	12.7 ±0.9	13.1 ±1.2	Aabc

Table 2. Effect of age and gender on BAT composition. Data are shown as mean ± SEM of 6-8 animals. Two-way ANOVA ($p < 0.05$): A, effect of age; G, effect of gender; AxG interaction of age and gender; NS, no significant difference. Post hoc analysis of age (LSD test; $p < 0.05$): a= 6 vs 18 mo, b= 6 vs 24 mo, c= 18 vs 24 mo. Student's t-test ($p < 0.05$): g= female vs. male.

145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4 , 3 mM MgCl_2 , 0.1 mM EGTA and 0.1 % BSA (pH 7.4 at 37°C). Non-phosphorylating (state 4) mitochondrial respiration rate was measured with pyruvate-malate (2.5 mM/2.5mM) as substrate in the absence and in the presence of 500 μM GDP in order to inhibit UCP1.

COX activity

Complex IV or COX (cytochrome c oxidase; EC 1.9.3.1) was measured using a spectrophotometric method [25]. Briefly, mitochondria were incubated in 0.1M NaPO_4H_2 , pH 7, in the presence of 2 $\mu\text{g}/\text{mL}$ catalase and 5 mM substrate DAB (3,3'-diaminebenzidine-tetrachloride). After 30 s, 100 μM reduced cytochrome c was added to start the reaction, and absorbance variation was followed over 15 min at 450 nm. COX activity was also measured in homogenate in order to calculate the total activity.

Total and mitochondrial DNA

Total DNA was measured in homogenates using the diaminobenzoic acid method [26]. Mitochondrial DNA was measured in pre-treated homogenates and mitochondrial fraction by real-time PCR as previously described [24] (primers sequence: 5'-TAC ACG ATG AGG CAA CCA AA-3'; 5'-GGT AGG GGG TGT GTT GTG AG-3'). Mitochondrial DNA was corrected by mg of total DNA to provide an estimation of the mitochondrial content per adipocyte.

Western blot for UCP1, COX IV and TFAM

As described elsewhere [27, 28], 15 μg mitochondrial protein (for UCP1 specific content) or 30 μg homogenate protein was fractioned by SDS-PAGE (12% polyacrylamide) and electrotransferred onto nitrocellulose filters. Ponceu S staining was used to provide visual evidence of correct loading and electrophoretic transfer of proteins to nitrocellulose filter. Membranes were incubated overnight at 4°C in a blocking solution of 5% non-fat powdered milk in T-PBS (phosphate-buffered saline, pH 7.5, containing 0.1% Tween 20). Antisera against UCP1 (UCP12-A, Alpha Diagnostics International, San Antonio, USA), COX subunit IV (MS407, MitoScience, Eugene, OR, USA), TFAM (supplied by Dr. Hidetoshi Inagaki), were used as primary antibodies. Developments of the immunoblots were performed using an enhanced chemiluminescence Western blotting analysis system (Amersham). Bands in films were analysed by scanner photodensitometry and quantified using Kodak 1D Image Analysis Software.

Thyroid and sex steroid hormone levels

Blood samples were allowed to clot and were centrifuged at 1000xg for 10 min to obtain serum. Samples were stored at -70°C until use. Serum T3, T4, testosterone, progesterone and 17 β -estradiol levels were measured using competitive immunoenzymatic colorimetric kits according to the manufacturers' protocols (Diametra, Milano, Italy).

Fig. 1. Mitochondrial DNA and protein content. A) Total mitochondrial DNA (mtDNA) was measured by real-time PCR in BAT homogenates and presented per mg of total DNA. B) Total mitochondrial protein (mtProt) was determined in mitochondrial fraction and presented per mg of total DNA. C) Ratio between mitochondrial protein and DNA content both measured in mitochondrial fraction. Data represent the mean $\pm$ SEM of 6-8 animals. Two-way ANOVA ($p < 0.05$): A, age effect; G, gender effect. Post hoc analysis (LSD test; $p < 0.05$): a = 6- vs 18-mo, b = 6- vs 24-mo, c = 18- vs 24-mo.

Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, version 14.0) was employed for all statistical analyses. Data are presented as means $\pm$ SEM. Differences between groups were assessed by two-way analysis of variance (ANOVA) followed by LSD post hoc test and Student's *t*-test. Results were considered statistically significant at the $p < 0.05$ level. Bivariate linear regression and ANOVA were used to assess the ability of serum hormone levels to explain the variance in the measured thermogenic parameters.

Result

Body, WAT and BAT weight

The evolution of body, white adipose tissue (WAT) and BAT weight with age in male and female rats is shown in Table 1. Body mass increased in both male and female rats, with female rats showing a lower increase between 6-mo and 24-mo of age (+18% females vs +42% males). Male rats showed a higher adiposity index (mg WAT/g body weight) than their age-matched female rats. Both genders showed an age-related increase in adiposity.

BAT mass in male rats showed a marked increase between 6-18-mo of age (+110%) and remained unchanged at 24-mo. In contrast, BAT of female rats showed increases at all ages (6-18 mo +76%, and 18-24 mo +32%). When adjusted to gram of body weight, BAT mass increased in males during the 6-18-mo period, indicating that BAT mass increased at a higher rate than body mass. However, female rats did not show differences in BAT/g body weight until 24-mo of age.

BAT composition

Table 2 shows the values of DNA, triglyceride and protein content in BAT. These parameters (per gram of

tissue) have also been normalized to mg of DNA in order to adjust the values per adipocyte. There was a progressive age-related decrease in the DNA content per gram of tissue, affecting both genders, which reached statistical significance when 24- and 6-mo-old rats were compared. However, total DNA, which illustrates the cellular content, was decreased only at 24-mo, reflecting that the net loss in adipocytes takes place after 18-mo of age.

Female rats showed a higher triglyceride content per gram of tissue than male rats but this difference disappeared when it was corrected per mg of DNA, indicating that there were no gender differences in fat content per cell. The magnitude of the triglyceride content per cell, together with its increase with age, suggests that the observed increase of BAT mass is mainly due to accumulation of lipids. In accordance with the gender differences found in BAT growth, the monthly calculated percentage of triglyceride accumulation was slightly higher in males (8 vs 6.8 %) during the 6-18-mo period whereas in the 18-24-mo period it was higher in females (7.3 vs 13.2 %).

Protein content per gram of tissue was found to be higher in female vs male rats at 6- and 18-mo. Protein decreased with age in both genders, but more markedly in females, converging at 24-mo of age. A similar pattern was observed when protein was adjusted per mg of DNA.

BAT mitochondrial DNA and mitochondrial protein

Mitochondrial content and function are the key parameters for BAT thermogenic capacity. In the present study we found that mtDNA per mg of total DNA, which illustrates the mitochondrial content per cell, was increased in both genders at 24-mo of age (Figure 1A). However, mitochondrial protein, which represents the

Fig. 2. Total COX activity and protein. Total brown adipose tissue COX activity (A) and total COX subunit IV protein content measured by Western blot (B). Data correspond to the mean $\pm$ SEM of 6-8 animals. Representative bands in film are shown. Two-way ANOVA ($p < 0.05$): A, age effect; G, gender effect. Post hoc analysis (LSD test; $p < 0.05$): a = 6- vs 18-mo, b = 6- vs 24-mo, c = 18- vs 24-mo. Student's t test ($p < 0.05$): g = male vs. female.

metabolic and thermogenic machinery in BAT, decreased in the 24-mo old rats (Figure 1B). The mtProtein/mtDNA ratio independently calculated from the isolated mitochondrial fraction, confirmed that protein content per mitochondria markedly decreased in 24-mo old rats (Figure 1C). On the other hand, the results also indicate that females showed a higher protein content per mitochondria at 6 and mo of age.

COX activity and protein levels

Cytochrome c oxidase or COX, which catalyzes one of the rate-controlling steps of mitochondrial respiration, is a good index of total BAT oxidative capacity. Thus, Figure 2 shows the COX activity and protein per gram of tissue. A similar pattern was found both in COX activity and COX protein levels, with 6-mo-old females showing greater activity and protein content than the similarly aged male rats. These gender differences disappeared in 18-mo-old rats as females decreased their COX activity but not their protein levels.

The major decrease in COX activity, paralleled by a drop in the levels of COX protein, was found in both genders at 24-mo of age. This significant loss in oxidative capacity is in accordance with the drop in mitochondrial protein previously shown (Figure 1B,C). No statistical significant changes were found in specific COX activity (per mg mitochondrial protein) (data not shown). Altogether, these data suggest that the main age-associated decrease in mitochondrial mass and oxidative capacity takes place after 18-mo of age.

Fig. 3. Mitochondrial respiration rate and UCP1 levels. Mitochondrial respiration rate (A) measured using pyruvate as substrate in the absence (closed bar) and in the presence (open bar) of 500 μ M GDP. Student's t test ($p < 0.05$): * -GDP vs +GDP. B) Specific and total UCP1 content in BAT measured by Western blot as described in Materials and Methods. Representative bands in films are shown. Data represent the mean $\pm$ SEM, with male levels set as 100%, of 6-8 animals per group. Two-way ANOVA ($p < 0.05$): A, age effect; G, gender effect. Post hoc analysis (LSD test; $p < 0.05$): a = 6- vs 18-mo, b = 6- vs 24-mo, c = 18- vs 24-mo. Student's t test ($p < 0.05$): g = male vs. female.

Fig. 4. Protein content of TFAM. Protein content of TFAM was measured by Western Blot in BAT homogenates and presented as arbitrary units per mg of protein. Representative bands in films are shown. Data represent the mean $\pm$ SEM, with male levels set as 100%, of 6-8 animals per group. Two-way ANOVA ($p < 0.05$): A, age effect. Post hoc analysis (LSD test; $p < 0.05$): a = 6- vs 18-mo, b = 6- vs 24-mo, c = 18- vs 24-mo.

	Male			Female			ANOVA
	6 mo	18 mo	24 mo	6 mo	18 mo	24 mo	
17 β -Estradiol (pg/ml)	20.3 $\pm$ 3.7	16.9 $\pm$ 2.5	15.7 $\pm$ 4.5	48.5 $\pm$ 7.7	28.9 $\pm$ 3.3	27.3 $\pm$ 3.9	G
Progesterone (ng/ml)	0.684 $\pm$ 0.233	0.798 $\pm$ 0.206	1.28 $\pm$ 0.37	33.4 $\pm$ 4.4	22.8 $\pm$ 6.13	23.2 $\pm$ 6.7	G
Testosterone (ng/ml)	1.30 $\pm$ 0.59	0.395 $\pm$ 0.070	0.401 $\pm$ 0.063	0.112 $\pm$ 0.027	0.095 $\pm$ 0.017	0.094 $\pm$ 0.023	G
T3 (ng/ml)	72.5 $\pm$ 5.5	77.6 $\pm$ 10.2	63.1 $\pm$ 4.7	97.5 $\pm$ 2.3 ^a	71.6 $\pm$ 5.44 ^a	63.6 $\pm$ 4.9 ^b	Ab, AxG
T4 (ng/ml)	402 $\pm$ 31	277 $\pm$ 46	268 $\pm$ 76	274 $\pm$ 44	206 $\pm$ 24	199 $\pm$ 49	Aab,G

Table 3. Serum T3, T4 and sex steroid levels. Serum total T3, T4 and sex steroid levels in 6, 18 and 24 mo-old male and female rats. Data are shown as mean $\pm$ SEM of 6-8 animals. Two-way ANOVA ($p < 0.05$): A, effect of age; G, effect of gender; AxG, interaction of age and gender; NS, no significant difference. Post hoc analysis of age (LSD test; $p < 0.05$): a= 6 mo vs 18, b= 6 mo vs 24 mo, c= 18 mo vs 24 mo. Student's t-test ($p < 0.05$): g= female vs. male.

	Parameters	17 β -Estradiol	Progesterone	Testosterone	T3	T4	T3/T4
Males	Hormones	T3/T4 0.499**	T3/T4 0.746**	-	-	-	17 β -Estradiol 0.499** Progesterone 0.746**
	Weight	-	BAT weight 0.481*	-	-	WAT weight -0.642**	BAT weight 0.597* BAT/ g bw 0.724**
	BAT composition	-	Trig/g BAT 0.722**	Protein/g BAT 0.696** Protein/mg DNA 0.584*	-	Trig/g BAT -0.714** Protein/mg DNA 0.639*	Trig/g BAT 0.799** Protein/mg DNA -0.538* DNA/depot 0.734**
	BAT function	-	-	-	-	-	-
	Hormones	T3 0.555**	Testosterone 0.823**	Progesterone 0.823**	T4 0.530* 17 β -Estradiol 0.555** BAT weight -0.650** Protein/g BAT 0.893** Trig/mgDNA -0.864** DNA/gBAT 0.777** COX/g tej 0.772** UCP1/g BAT 0.869** MMR 0.593*	T3 0.530*	-
Females	Weight	-	-	-	-	-	-
	BAT composition	-	-	-	-	-	-
	BAT function	-	MRR GDP 0.562*	MRR GDP 0.532*	-	-	mtProt/mt DNA 0.712**
	Hormones	T3 0.412* T3/T4 0.356* Progesterone 0.568**	17 β -Estradiol 0.568**	T4 0.434*	17 β -Estradiol 0.412* Protein/g BAT 0.718**	Testosterone 0.434*	17 β -Estradiol 0.356*
Whole group	Weight	BAT weight -0.504**	BAT weight -0.416*	BAT/g bw -0.344*	Body weight -0.400*	-	-
	BAT composition	Protein/g BAT 0.545** DNA/g BAT 0.475**	Protein/g BAT 0.388* Trig/g BAT 0.385*	-	Trig/mg DNA -0.536** DNA/gBAT 0.619**	Trig/g BAT -0.498** Protein/mg DNA 0.396*	Trig/g BAT 0.585** DNA/depot 0.500**
	BAT function	COX/g BAT 0.406* UCP1 0.444*	COX/g BAT 0.449*	-	COX/g tej 0.553** UCP1/g BAT 0.680** MMR 0.574**	-	mtProt/mtDNA 0.391*
	Hormones	T3 0.412* T3/T4 0.356* Progesterone 0.568**	17 β -Estradiol 0.568**	T4 0.434*	17 β -Estradiol 0.412* Protein/g BAT 0.718**	Testosterone 0.434*	17 β -Estradiol 0.356*

Table 4. Linear bivariate regression analysis. Table showing the best correlations between BAT parameters and serum hormone levels. Values are Pearson's correlation coefficient (r). All experimental measures obtained in BAT were pooled in a database and analyzed for correlation with serum levels of T3, T4, sex steroids as well as T3/T4 ratio. Analysis was performed in males and females separately (n=22) and whole experimental group (n=44). Only significant correlations (* $p < 0.05$, ** $p < 0.01$) are shown. Trig, Triglycerides; MRR, mitochondrial respiration rate; mtProt, mitochondrial protein; bw, body weight.

Mitochondrial respiration rate and UCP1 protein levels

In order to study the UCP1 activity and levels in BAT we used mitochondrial state 4 respiration rate in the absence and in the presence of GDP (specific UCP1 inhibitor) as well as Western blot analysis on mitochondrial fraction and tissue.

As shown in Figure 3A, 6- and 18-mo-old female rats showed higher oxygen consumption per mg of mitochondrial protein than male rats, although it did not reach statistical significance ($p = 0.07$). Age-related reduction in

oxygen consumption was significant at 18-mo of age in males, whereas this drop was not observed until 24-mo of age in females. The GDP inhibitory effect over respiration rate indicated that uncoupling activity was decreased earlier in males (18-mo) than in females (24-mo). No gender differences were observed in UCP1-inhibited respiration (in the presence of GDP) at any of the studied ages.

Although UCP1 activity was decreased with age, the UCP1 content per mitochondrial protein was unchanged, showing only an age-related increase in vari-

Fig. 5. Linear regression analysis. Correlations of T3 serum levels with UCP1 (A) and mitochondrial respiration rate with substrate alone (MRR) (B) or +GDP (MRR_{GDP}) (C). Linear regression analysis was made separately for each gender (n=22). Different age groups were pooled for the analysis. Open and closed squares represent males and females. ANOVA * p<0.05, ** p<0.01.

ability (Fig 3B). This discordance between UCP1 activity and protein levels has been previously reported [6] and may denote age-related increases in UCP1 inhibition. On the other hand, total UCP1 content per gram of BAT showed a similar pattern to mitochondrial respiration rate, indicating a concordance between the response of UCP1 at activity level as well as total content level.

TFAM protein levels

TFAM has been identified as key regulator of mitochondrial differentiation and proliferation [29]. As shown in Figure 4, 24-mo-old rats showed a decrease in TFAM levels which was more marked for males.

Serum total T3, T4 and sex steroid levels

As expected, 17 β -estradiol, progesterone and testosterone showed a marked gender dimorphism with higher levels of 17 β -estradiol and progesterone in females and testosterone in males in all the age groups studied (Table 3). These sexual steroid hormones in their respective gender were markedly decreased in old (18-mo and 24-mo) compared to young animals (6-mo), reflecting the age-associated decline in gonadal function.

Serum total T3 was higher in 6-mo-old female rats than in their male counterparts. This gender difference disappeared at 18-mo of age with females decreasing T3 levels, whereas the male levels were unchanged with age. Serum total T4 showed a similar decline with age which was more noticeable in males. In relation to gender, young males showed significantly higher levels of T4 than females and maintained this tendency with advancing age.

Bivariate analysis regression

Growth, differentiation and thermogenesis, three important processes which involve the mitochondria, are affected by steroid and thyroid hormones in BAT. In view of the gender differences found in steroid and thyroid

status we decided to employ correlation analysis to estimate the ability of these hormones to explain the variance during the aging process in the BAT thermogenic capacity of male and female rats. Thus, Table 4 compiles the variables of the study which were better correlated with each hormone in each gender and whole experimental group. T3 was the hormone which showed better correlations with the most important thermogenic parameters such as UCP1, COX activity, mitochondrial respiration rate and BAT protein levels. These correlations were strongly significant in females and maintained statistical significance when data from males were included. As shown in Figure 5, UCP1 levels and mitochondrial respiration rate were closely related with T3 levels in females (Fig 5AB), whereas this correlation was lost when the UCP1 was inhibited by GDP (Fig 5C). This suggests a strong relation between T3 and the gender differences found in uncoupling activity.

Female sex steroid hormones (17 β -estradiol and progesterone) showed stronger correlations with BAT thermogenic parameters than the male sex steroid testosterone. Between the hormones themselves, there was correlation in some cases between 17 β -estradiol, progesterone and T3 or T3/T4 ratio. T3/T4 ratio was also found to correlate in males with BAT mass and composition, whereas in females it was significantly correlated only with mtProt/mtDNA ratio, which reflects degree of mitochondrial differentiation. All in all, these correlations suggest that T3 is closely related with the BAT thermogenic decline in females.

Discussion

With the onset of aging, thermogenesis and energy expenditure decline in humans and laboratory animals, resulting in an inability to maintain thermal homeostasis

during cold exposure and also contributing to the age-related increase in adiposity [4, 30]. Among other factors, the decline in thermal homeostasis has been associated with lower BAT recruitment [3, 5, 31], a term that refers to the thermogenic/metabolic capacity of the tissue. However, older female rats, in contrast to males, have been shown to maintain a higher BAT thermogenic capacity, suggesting the existence of a sexual dimorphism in the effect of aging on the functional decline of BAT [5].

In the present study, maximal gender differences in recruitment parameters were found in younger animals (6-mo), with female rats showing higher total and mitochondrial protein content, UCP1 content and greater BAT size in relation to body weight. In agreement, previous works have shown, by electron microscopy analysis of BAT, that young female rats have highly differentiated mitochondria with larger size and higher cristae length and density [23, 32]. These differences in recruitment state under usual rodent housing temperature (22 °C) have been attributed to gender differences in cold sensitivity [33].

With advancing age, male and female rats showed a gender-dependent decline in their thermogenic function, with males showing an earlier decrease in BAT thermogenic activity than female rats. Age-related increase in BAT mass was higher in males between 6- and 18-mo of age, probably by means of a higher rate of fat accumulation, a sign of BAT atrophy. According with this earlier attenuation of BAT energy expenditure in males, body weight and fat accumulation were found to be higher in old male compared to female rats (e.g. 1.6% monthly increase in body weight of males vs 0.5 % of females from 6 to 18-mo of age). However, although BAT thermogenesis is an important component of energy balance, the involvement of other components such as physical activity, resting metabolic rate or food intake may not be ruled out.

From a biochemical point of view, the decrease in BAT thermogenic capacity of 18-mo-old males was not related with a loss in mitochondria number or oxidative capacity, since mitochondrial protein content, mtDNA and COX protein were similar between 6- and 18-mo old rats. Instead, the main change found was a loss in the mitochondrial UCP-dependent respiration rate, i.e. a decrease in uncoupling activity. Strangely, UCP1 content in the fraction was similar between 6- and 18-mo old rats, a fact that was previously observed in male rats by other authors [6]. Giving a further step, our study performed in male and female rats indicate that this 'inhibition' of UCP1

would take place first in males, with this drop in UCP1 activity not present in females until the next age studied (24-mo). Although endogenous free fatty acids in isolated mitochondrial fraction are usually enough to allow UCP1 activity, it is worth noting that we did not add free fatty acids in our activity assay media. Thus, it must be taken into account that UCP1 activity may not be maximal.

BAT was markedly atrophied in the oldest groups (24-mo) of male and female rats used in this study. This loss in BAT functionality consisted of a great accumulation of fat together with decreases in cell number (total DNA) and mitochondrial recruitment (protein content, COX activity and UCP1). However, mitochondrial number, reflected by mtDNA content, was significantly increased in 24-mo-old rats of both genders, suggesting an age-induced proliferation of mitochondria. Similar increases in the amount of mtDNA with age has been described in rodents in other organs such as brain, heart, lung, skeletal muscle, kidney and liver [34, 35]. In accordance with previous works [36, 37] the poor mitochondrial machinery found in the tissue of 24-mo-old rats suggests that the increase of mitochondrial proliferation with age is probably a compensatory mechanism for the defective mitochondria. Along these lines, the expression patterns of TFAM were found to be opposite to the age-related pattern of mtDNA content. TFAM plays a key role in mitochondrial biogenesis since it is basic to initiation of both replication and expression of mtDNA [7, 8]. Although the precise mechanism by which TFAM controls mtDNA replication and transcription are not fully understood, some reports have pointed out that mtDNA replication can be initiated by small amounts of TFAM, whereas expression of mtDNA, which is related to mitochondrial differentiation, is activated only at high concentrations of this factor [38-40]. Taking this into account, the decrease in TFAM levels in BAT of 24-mo-old rats may be in accordance with both the age-related increase in mitochondrial proliferation (mtDNA) and the lower degree of mitochondrial differentiation observed in these animals.

The observed gender differences in thermogenic decline could be dependent, at least in part, on the action of sex steroid hormones. The reproductive system is one of the first systems to show age-related decline in function, with altered sex hormonal status in females in most species long before the end of the life span. In our study, female rats showed a decrease in 17 β -estradiol and progesterone at 18-mo of age, the time by which rats become acyclic (17-20 months) [41, 42]. There are abun-

dant reports in the literature considering sex steroid hormones as a potential modulator of BAT thermogenesis [12, 15, 16]. In our study, when both sexes were considered, sex steroid hormones were well correlated with BAT composition and thermogenic parameters, especially 17 β -estradiol and progesterone. However, when the analysis was split by gender, the results indicated that the active form of thyroid hormone, T3, has the highest potential for explaining the gender-dependent variation of BAT parameters. Thus, T3 showed strong correlations in females with most of the BAT parameters, including those of function, and 17 β -estradiol was found to correlate only with T3 (or T3/T4) levels. These observations suggest that T3 may mediate, at least in part, the effects of gender on functional decline in BAT during aging. In agreement, many reports in the literature support the existence of gender differences in thyroid status. Several authors have also found higher serum T3 levels in females [43-45] as well as greater thyroxine 5'-deiodinase (T5'D) activity in BAT and other tissues [19, 46]. Sex hormones have also been shown to influence thyroid function, with estrogens modulating the pituitary parameters involved in the regulation of TSH secretion [20]. Moreover, it must be taken into account that T3 is the major regulator of oxidative metabolic processes, regulating the expression of enzymes and other factors involved in oxidative metabolism [11]. In BAT, T3 promotes UCP1 synthesis at transcriptional level as well as UCP1 activity by modulation of cAMP production [47]. Thus,

thyroid hormones must be considered a candidate factor underlying the gender differences in BAT. Future work along these lines may contribute to the understanding of the gender differences in the age-related decline of physiological functions.

On the whole, our results suggest that female rats show a greater recruitment in BAT under conditions of mild-stimulation of thermogenesis (22 °C) and have a higher ability to preserve uncoupling activity, at least until the decline in reproductive function, after which both genders undergo an intense BAT atrophy. 17 β -estradiol, progesterone and T3 are closely related with the gender differences in BAT decline during aging, which suggests a mediating role for T3 in the gender effects on BAT functionality.

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