It's about time: Circadian rhythm and metabolism

Schilperoort, M.

Testosterone reduces brown fat activity in mice

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Abstract

Brown adipose tissue (BAT) protects against hypothermia by burning substantial amounts of glucose and lipids to produce heat, a process called non-shivering thermogenesis. Interestingly, BAT activity and core body temperature are lower in males as compared to females. It has been suggested that variation in androgen levels could be responsible for this sex difference, although current evidence is conflicting. In this study, we aimed to further investigate the role of androgens in BAT activity. We found that depletion of androgens in mice by surgical castration promotes the expression of markers for lipolysis and thermogenesis in BAT. While lipid uptake was increased in BAT from castrated mice, lipid content was decreased, collectively confirming increased BAT activity. The increased BAT activity in castrated mice was reversed by testosterone supplementation, but was not dependent on androgen receptor expression in BAT. Activation of the androgen receptor also did not diminish the activity of cultured brown adipocytes. Instead, noradrenaline levels were increased in BAT from castrated mice, suggesting that androgens may increase sympathetic outflow to BAT. Altogether, our results demonstrate that androgens are potent negative regulators of BAT activity and could be responsible for sex differences in human BAT activity.

Introduction

Maintenance of core body temperature is crucial for cellular function and organism survival. Yet, homeostatic body temperature shows certain variation. For example, body temperature is affected by sex, as illustrated by a higher body temperature in female mice as compared to male mice [1]. This sex difference is abolished by surgically removing the testes (i.e. castration) of male mice, which raises core body temperature to similar levels as in female male [1, 2]. These results support a role of the male sex hormones, or androgens, in the regulation of core body temperature.

Brown adipose tissue (BAT) helps to maintain core body temperature through non-shivering thermogenesis [3]. Active BAT takes up high amounts of nutrients such as glucose and fatty acids (FAs) from the circulation to fuel mitochondrial oxidation. Mitochondrial oxidation in brown adipocytes is uncoupled from ATP generation by uncoupling protein 1 (UCP1), and therefore energy from the oxidation of nutrients is released as heat. Interestingly, the thermogenic capacity of BAT is affected by sex. BAT from male rats shows lower UCP1 expression and mitochondrial respiration as compared to BAT from female rats [4], and in humans, BAT activity as assessed by radiolabeled glucose uptake is lower in men as compared to women [5-7].

Despite the evidence mentioned above, relatively little is known about a potential role of androgens in BAT activity and existing data is conflicting. Depletion of androgens by castration has been shown to decrease BAT weight [8], possibly due to an increased lipolytic activity of BAT resulting in depletion of intracellular lipid stores [9]. In line with this, activation of the androgen receptor (AR) by the natural agonist dihydrotestosterone (DHT) increases BAT weight [8], suggestive of a reduced BAT activity. This effect could be mediated by a direct effect of AR signaling on BAT, as brown adipocytes clearly express the AR [10]. Indeed, treatment with testosterone inhibits the activity of brown adipocytes *in vitro* by downregulating thermogenic genes (e.g. *Ucp1* and *Pgc1a*) and diminishing lipolysis [11-13]. These results point towards a negative effect of androgens on BAT activity. However, this appears to contradict with the decreased *Ucp1* expression that is observed in BAT of AR-deficient mice [14, 15].

In the current study, we aimed to further elucidate the role of androgens in BAT activity. We set out to compare FA uptake by BAT, one of the best proxies for metabolic BAT activity *in vivo* [16, 17], between sham-operated and castrated mice. Furthermore, we examined whether the effects of castration on BAT are mimicked by BAT-specific AR-deficiency, to investigate whether androgen depletion affects BAT activity via direct or indirect mechanisms.

Materials and Methods

Animals

To study the effect of castration on BAT activity, 10 week-old male C57Bl/6J mice (Charles River Laboratories) were randomized into three experimental groups based on body weight and fat mass: (1) sham operation with vehicle injection, (2) castration with vehicle injection, or (3) castration with testosterone injection (n = 8 mice per group). From the day of sham operation or castration onwards, mice were subcutaneously injected with either vehicle or testosterone (2.5-3.0 mg/kg; Sigma T1875) in an attempt to restore endogenous testosterone levels. Injections were given once every three days, 2 h before onset of the dark phase. After three weeks (one day after the last injection), mice were killed to evaluate uptake of radiolabeled triglyceride (TG)-derived fatty acids (FAs).

To study the role of the AR in BAT activity, male BAT-specific AR-deficient mice (BAT-AR$^{-/y}$) were generated by breeding AR$^{flox/+}$ female mice (from Dr. Verhoeven, Katholieke Universiteit Leuven, Belgium) with B6.FVB-Tg(Ucp1-cre)1Evdr/J male mice (Jacksson Laboratories). The AR is located on the X chromosome, and male mice with the genotype AR$^{flox/y}$ Ucp1-Cre$^{+/-}$ are BAT-AR$^{-/y}$; littermate controls are AR$^{+/y}$ Ucp1-Cre$^{+/-}$ (BAT-AR$^{+/y}$). BAT-AR$^{-/y}$ and BAT-AR$^{+/y}$ mice were castrated or sham-operated at 10 weeks of age. At 24 weeks of age mice were killed to evaluate uptake of radiolabeled TG-derived FAs.
All mice were housed in conventional cages with a 12 h light/dark cycle and *ad libitum* access to chow diet and water. Experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals after having received approval from either the Leiden University Ethical Review Board or the Ethics Committee on Animal Care and Use in Gothenburg

Castration

Mice were anesthetized with isoflurane and either sham-operated or bilaterally castrated using a cauterizer (Fine Science Tools). Buprenorphine (Temgesic) was used for analgesia after the procedure.

Food intake, body weight and body composition measurements

Food intake and body weight of mice were measured with a scale, and lean and fat mass with an EchoMRI-100-analyzer (EchoMRI).

Tissue histology and immunohistochemistry

Interscapular BAT (iBAT) was cut into 10 µm cryosections and stained with hematoxylin and eosin (H&E) using standard protocols. The area of intracellular lipids was quantified by a blinded observer using Visiopharm Integrator System (Version 5.3.0.1562, Visiopharm). Immunofluorescent staining of UCP1 was performed by using a polyclonal rabbit anti-mouse antibody against UCP1 (Abcam, ab23841; 1 µg/mL), followed by a secondary antibody AF595-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, 711-587-003; 1 µg/mL), and nuclei were stained with DAPI (Sigma-Aldrich, D9542). UCP1-positive area was quantified by a blinded observer using Visiopharm Integrator System (Version 5.3.0.1562, Visiopharm).

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted from iBAT with RNeasy Plus Universal Mini Kit (Qiagen, 73404) according to the manufacturer's instructions. cDNA was synthesized from total RNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, 4374966). RT-PCR analysis was done with predesigned TaqMan Gene Expression Assays (Applied Biosystems). Analyses were performed using a Viia 7 Real-time PCR System (Thermofisher Scientific), and expression levels of genes of interest were normalized to expression of the housekeeping gene *18s*.

Uptake of radiolabeled triglyceride-derived fatty acids

Glycerol tri[^{3}H]oleate-labeled lipoprotein-like TG-rich emulsion particles (80 nm) were prepared and characterized as described previously [18]. Mice were fasted for 4 h and injected with 200 µL of emulsion particles (1 mg TG per mouse) via the tail vein, 2 h before onset of the dark phase. After 15 min, mice were killed by cervical dislocation and perfused with ice-cold PBS through the heart. Thereafter, organs were harvested and weighed, dissolved overnight at 56°C in Tissue Solubilizer (Amersham Biosciences), and mixed with Ultima Gold scintillation liquid

(PerkinElmer). The uptake of glycerol tri[^{3}H]oleate-derived radioactivity was quantified and expressed per gram of wet tissue weight and/or per organ.

Cell culture and experiments

Immortalized preadipocytes were generated and cultured as previously described [19]. Preadipocytes were differentiated for 14 days. During the last 3 days of differentiation, cells were grown in medium containing hormone-deprived (charcoal-stripped) serum. Cells were treated with DHT (10 nM) or vehicle for 8 h, followed by stimulation with noradrenalin (NA; 1 μM) or vehicle for 16 h. Medium was collected to measure glycerol concentrations using a commercially available assay (Instruchemie, Delfzijl, The Netherlands), and cells were harvested for gene expression analysis. To determine the oxygen consumption ratio (OCR), cells were differentiated in 96-well Seahorse Bioscience assay plates for measurement using the Seahorse Bioscience XF96 extracellular flux Analyzer (Seahorse Bioscience). After differentiation, cells were pre-treated with DHT (10 nM) or vehicle for 8 h before starting Seahorse measurements. NA or vehicle were preloaded in the reagent delivery chambers and pneumatically injected into the wells after five baseline measurements (to a final concentration of 1 μM). Cellular OCR was measured in real-time every 8 min.

Tissue noradrenalin measurements

NA levels in one lobe of iBAT were determined by high-performance liquid chromatography (HPLC) followed by electrochemical detection. The tissue was kept cold and homogenized 3 x 20 sec in 500 μL homogenization solution (5 mM EDTA in 0.1 M percloric acid) using an ultra-sonifier (Branson Ultrasonic Sonifier 250). After centrifugation (12,000 g, 4°C, 10 min), the middle layer was transferred to a new tube and 50 μL trichloroacetic acid was added followed by another round of centrifugation (12,000 g, 4°C, 10 min). The supernatant was filtered (0.22 μm syringe filter, Nylon syringe filters, Skandinaviska Genetec AB) and diluted in milliQ water. NA was analyzed on an reversed phase column (Capcell Pak 50 x 2 mm, 3 μm C18; Phenomenex, operated at 30°C) with a mobile phase (flow rate 0.3 mL/min) consisting of 150 mM NaH2PO4, 4.76 mM citric acid, 3 mM sodium dodecyl sulphate, 50 μM EDTA, as well as 10% MeOH and 15% acetonitrile. NA was detected by electrochemical detection (ESA Coulochem, Thermo Fisher Scientific) operated at 220 mV versus the cell. An external standard containing 2.95 fmol/μL NA was used to identify and quantify NA content in the sample (Chromeleon software, Thermo Fisher Scientific).

Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed using the Student's T-test, one-way ANOVA, or two-way ANOVA as indicated in the figure legends using GraphPad Prism (version 7.02). Differences between groups were considered statistically significant at $P < 0.05$.

Results

Castration increases markers of lipolysis and thermogenesis in BAT

Castration (i.e. orchiectomy (ORX)) markedly reduced BAT weight as compared to sham-operation (Fig. 1A). This effect was already observed after 6 days, but most pronounced after 21 days. After 21 days, we also observed an increased expression of genes involved in lipid metabolism (*Elovl3, Lpl*) and thermogenesis (*Pgc1a, Ucp1*) in BAT from ORX mice as compared to sham controls (Fig. 1B). Histological quantification revealed a reduced lipid content (Fig.

1C), and immunofluorescent stainings demonstrated an increased UCP1 expression (Fig. 1D) in BAT from ORX mice. These results suggest that androgen depletion increases the lipolytic and thermogenic capacity of BAT.

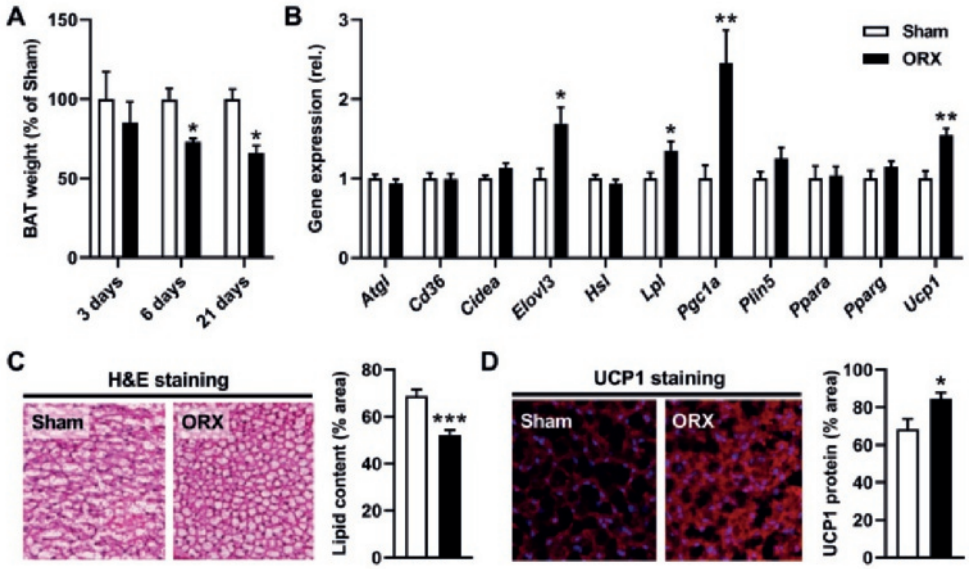

Figure 1. Castration reduces BAT weight and lipid content, and increases the expression of thermogenic markers. Sham-operated (Sham) and castrated (ORX) mice were killed after 3, 6 or 21 days following the surgical procedure (n = 4-9 per group), and interscapular BAT (iBAT) was collected and weighed (A). After 21 days, mRNA was isolated from iBAT and expression of genes involved in BAT activity was measured (B). Sections of iBAT were stained with haematoxylin and eosin (H&E), and lipid content of BAT was quantified (C). An immunofluorescent staining of UCP1 was performed on iBAT sections, and the UCP1-positive surface area was quantified (D). Data represent means ± SEM. *P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001 compared to the sham group, according to the Student's T-test.

Castration reduces body weight and increases fatty acid uptake by BAT by depleting testosterone

To further investigate whether androgen depletion affects energy metabolism and BAT activity, we studied three groups of mice: (1) sham-operated, (2) castrated, and (3) castrated and supplemented with testosterone (ORX + Test). ORX mice demonstrated a reduced body weight gain from week 1 onwards, which was reversed upon testosterone treatment (Fig. 2A). Castration reduced both lean mass (Fig. 2B) and fat mass (Fig. 2C) after 1 week, while long-term castration primarily reduced lean mass. Food intake was not different between the groups (Fig. 2D), indicating that the weight loss observed in ORX mice is most likely the result of an increased energy expenditure. The weight of seminal vesicles – a biomarker for androgen exposure – was determined to validate the different interventions. As expected, ORX markedly reduced seminal vesicle weight which was restored with testosterone supplementation (Fig. 2E).

Weights of metabolic organs such as the gonadal white adipose tissue (gWAT), liver, and both interscapular and subscapular BAT (iBAT and sBAT, respectively) were also reduced in ORX mice as compared to sham controls. Weight of the gWAT was only partially restored by testosterone treatment, but weight of the liver, iBAT and sBAT was fully restored by re-establishing testosterone levels.

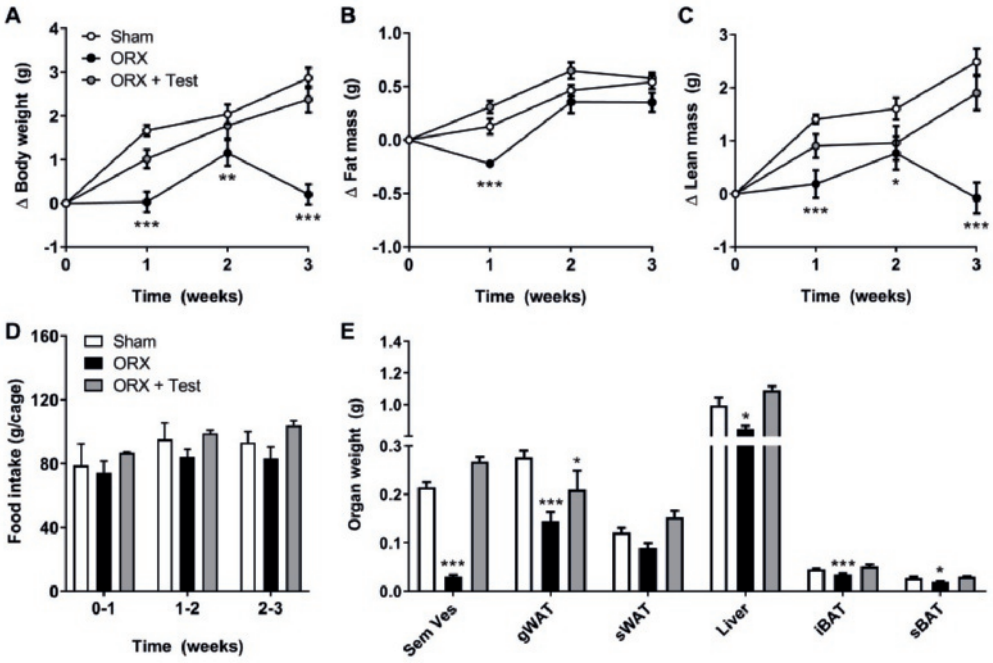

Figure 2. Castration reduces total body weight due to an initial loss of fat mass and prolonged loss of lean mass. Body weight (A), lean mass (B) and fat mass (C) were measured at indicated timepoints and expressed as compared to baseline (delta) in sham-operated mice (Sham), castrated mice (ORX), and castrated mice supplemented with testosterone by subcutaneous injection (ORX + Test) (n = 8 per group). Food intake was measured weekly and expressed as gram per cage (D). After three weeks, mice were killed and weight of the seminal vesicles, gonadal WAT (gWAT), subcutaneous WAT (sWAT), liver, interscapular BAT (iBAT) and subcutaneous BAT (sBAT) was measured. Data represent means ± SEM. *P $<$ 0.05, **P $<$ 0.01, ***P $<$ 0.001 compared to the sham group, according to two-way ANOVA with Dunnett's post hoc test.

To evaluate tissue-specific uptake of FAs, mice were intravenously injected with TG-rich lipoprotein-like particles labeled with glycerol tri[^{3}H]oleate prior to being killed. Castration increased the uptake of [^{3}H]oleate per gram of iBAT and sBAT by more than 3-fold (Fig. 3A), indicating highly increased metabolic BAT activity. This effect was still present when correcting for the reduced whole organ weights (Fig. 3B). Testosterone supplementation completely abolished the increased FA uptake by BAT observed in ORX mice, to the same levels as in sham-operated control mice.

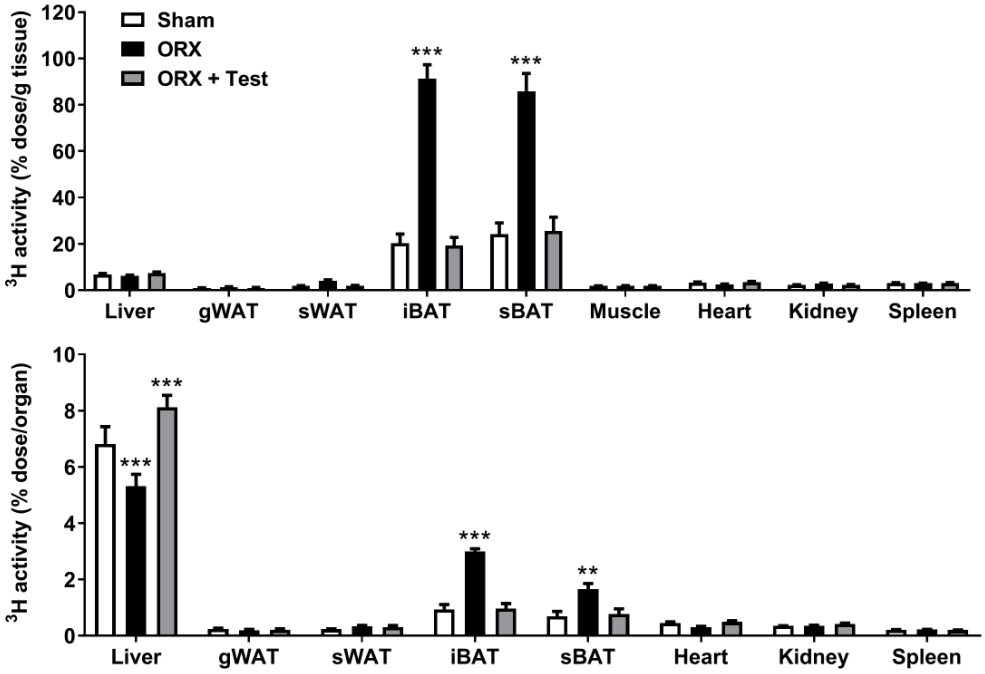

Figure 3. Castration increases the uptake of triglyceride-derived fatty acids by BAT. Sham-operated mice (Sham), castrated mice (ORX), and castrated mice supplemented with testosterone by subcutaneous injection (ORX + Test) (n = 8 per group) were intravenously injected with glycerol tri[3H]oleate-labeled TG-rich lipoprotein-like emulsion particles. After 15 min, mice were killed and uptake of glycerol tri[^{3}H]oleate-derived radioactivity per gram of wet tissue (A) or whole organ (B) was determined in various organs, including gonadal WAT (gWAT), subcutaneous WAT (sWAT), interscapular BAT (iBAT) and subscapular BAT (sBAT). Data represent means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the sham group, according to two-way ANOVA with Dunnett's post hoc test.

The effect of castration is not dependent on AR expression in BAT

Next, we aimed to evaluate whether activation of BAT by androgen depletion is mediated via AR signaling in brown adipocytes. The AR agonist DHT did not affect basal or NA-induced respiration (Fig. 4A), nor did DHT affect basal or NA-stimulated glycerol release (Fig. 4B), a measure of intracellular lipolysis. While brown adipocytes did show a classical response to NA by upregulating Ucp1 gene expression, DHT did not affect expression of *Ucp1* (Fig. 4C), *Pgc1a* (Fig. 4D), *Adrb3* (Fig. 4E) or *Lpl* (Fig. 4F).

As the *in vitro* data suggest that modulation of AR signaling in BAT may not be responsible for the castration-induced metabolic phenotype, we next aimed to confirm this in an *in vivo* setting. BAT-specific AR-deficient mice (BAT-AR$^{-/y}$) and wildtype littermates (BAT-AR$^{+/y}$) were sham-operated or castrated, followed by the evaluation of tissue-specific uptake of FA upon injection of glycerol tri[^{3}H]oleate-labeled particles. The weight of iBAT (Fig. 5A) and sBAT (Fig. 5B) was not different between BAT-AR$^{-/y}$ mice and wildtype littermates, and castration similarly reduced BAT weight in both genotypes. FA uptake per gram iBAT (Fig. 5C) and sBAT (Fig. 5D) was also not affected by genotype, and increased by castration independent

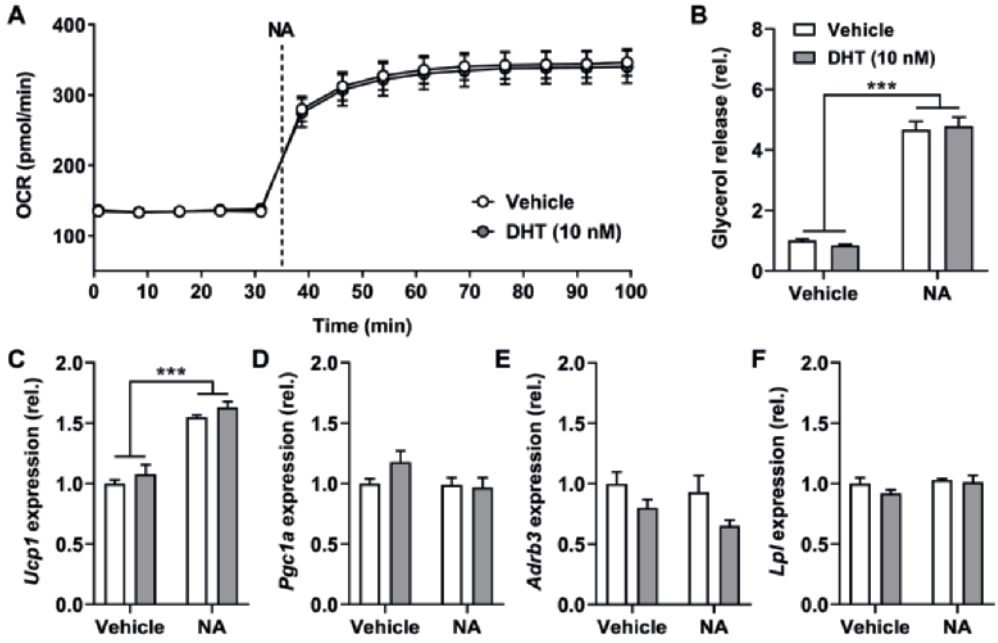

Figure 4. AR agonism does not increase brown adipocyte activity *in vitro*. Immortalized brown adipocytes (n = 19-22 wells per group) were treated with vehicle or DHT (10 nM) for 8 h, after which the oxygen consumption rate (OCR) was measured in a Seahorse XF96 Analyzer (A). After five baseline measurements, either vehicle or NA (to a final concentration of 1 μM) was injected into the wells. Brown adipocytes (n = 3 per group) were treated with vehicle or DHT (10 nM) for 8 h, followed by an additional stimulation with vehicle or NA (1 μM) for 16 h. Hereafter, culture medium was collected to measure glycerol concentrations (B) and cells were harvested to analyze gene expression of *Ucp1* (C), *Pgc1a* (D), *Adrb3* (E), and *Lpl* (F). Data represent means ± SEM. ***P $<$ 0.001 of the vehicle treated groups compared to the NA treated groups, according to two-way ANOVA with Sidak's post hoc test.

of genotype. Altogether, these results demonstrate that the increased BAT activity observed in ORX mice did not result from relieving a direct inhibitory effect on AR signaling in brown adipocytes.

Castration increases sympathetic outflow to BAT

ORX mice demonstrated a rapid and persistent loss of lean mass (Fig. 2C), likely due to muscle wasting as a result of androgen deficiency. Therefore, decreased thermogenesis in muscle may be compensated for by an increased thermogenesis in BAT, mediated by an increased sympathetic outflow to BAT. To explore this possibility, we measured NA levels in BAT of sham-operated and ORX mice. NA levels were increased in BAT of ORX mice as compared to sham controls after already 6 days, although this effect was abolished by correcting for the weight of the measured lobe of BAT (Fig. 6A). After 21 days, NA levels per mg BAT were more than 2-fold higher in ORX mice, and still tended to be higher when correcting for the BAT weight (Fig. 6B). Thus, androgen depletion by castration may increase sympathetic outflow towards BAT, thereby stimulating its thermogenic activity.

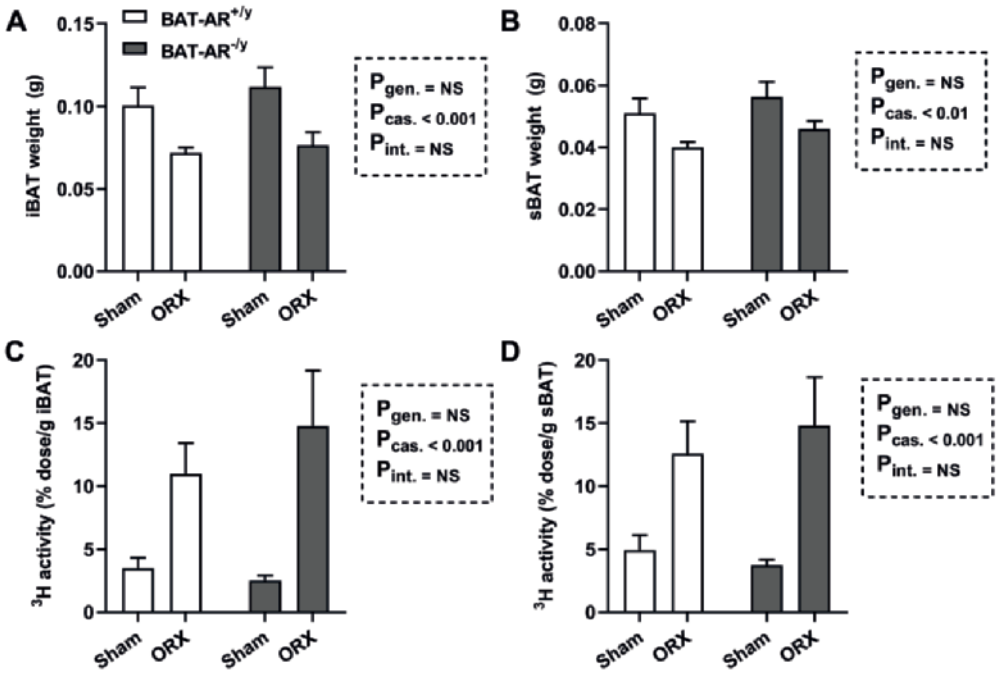

Figure 5. AR expression does not affect basal or castration-induced BAT activity *in vivo*. BAT-specific AR-deficient mice (BAT-AR$^{-/y}$) and wildtype littermates (BAT-AR$^{+/y}$) were sham-operated (Sham) or castrated (ORX) (n = 9-12 per group). After 21 days, mice were killed and the interscapular BAT (iBAT; A) and subscapular BAT (sBAT; B) were collected weighed. Mice were intravenously injected with glycerol tri[^{3}H]oleate-labeled TG-rich lipoprotein-like emulsion particles 15 min before sacrifice, and uptake of glycerol tri[^{3}H]oleate-derived radioactivity per gram of iBAT (C) and sBAT (D) was determined. The data was analyzed by using two-way ANOVA, and P-values of the effects of genotype ($P_{gen.}$), castration ($P_{cas.}$) and the interaction between genotype and castration ($P_{int.}$) are stated in a box next to each subfigure. Data represent means ± SEM.

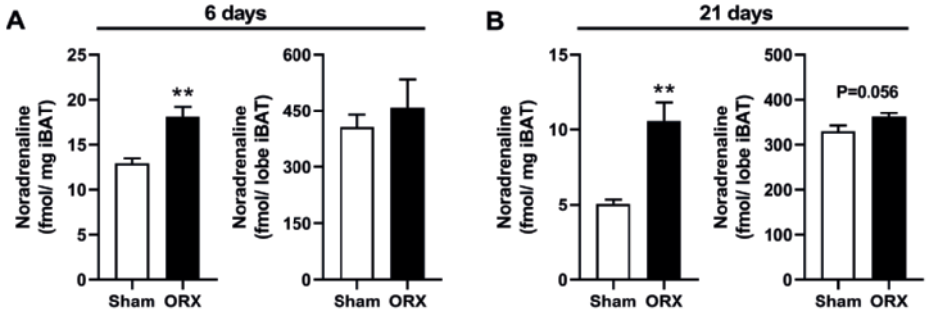

Figure 6. Castration increases NA levels in BAT. Noradrenalin (NA) levels per mg or lobe of interscapular BAT (iBAT) were measured by HPLC in sham-operated (Sham) and castrated (ORX) mice killed after 6 days (A) or 21 days (B) following surgical procedure (n = 7-9 per group). Data represent means ± SEM. **P $<$ 0.01 compared to the sham group, according to the Student's T-test.

Discussion

In this study, we explored the potential role of androgens in BAT activity. We demonstrated that androgen depletion by castration of mice strongly increases BAT activity. Mechanistically, this effect is independent of AR expression on BAT, and possibly explained by an increased sympathetic outflow to BAT.

Our findings that castration of male mice reduces body weight gain and lowers fat mass, at least on the short-term, confirm previous observations by others [2, 20, 21]. An increased BAT activity has been suggested to underlie these metabolic effects, as castration increases body temperature and upregulates *Ucp1* expression in BAT [2]. We now demonstrate that castration not only increases the expression of lipolytic and thermogenic genes in BAT, but also increases TG-derived FA uptake by BAT as a direct indicator of an increased metabolic BAT activity. It is likely that this increase in BAT activity promotes energy expenditure, as castration lowered body weight without reducing food intake. Thus, androgens appear to be potent negative regulators of BAT activity and energy metabolism.

Previous studies suggest that AR signaling in BAT directly impairs brown adipocyte activity. Using murine brown adipocytes, as little as 1 nM of testosterone has been reported to reduce expression of *Pgc1a*, an important regulator of mitochondrial biogenesis and thermogenic function, and decrease glycerol release [11, 12]. Treatment of brown adipocytes with 100 nM of testosterone also reduced expression of *Ucp1*, an effect that was blocked by the AR antagonist flutamide [13]. We could not replicate these effects of AR activation with DHT, an agonist that is about 2- to 3-fold more potent as compared to testosterone [22]. Also, we did not find any effect of DHT on cellular respiration, a direct measure of brown adipocyte activity. We are not the first ones to have difficulties in reproducing the reported inhibitory actions of AR signaling in brown adipocytes, and others have even reported that AR signaling can promote *Ucp1* transcription [14]. These results question whether androgen depletion by castration stimulates brown fat activity by diminishing AR signaling in brown adipocytes.

Global AR-deficient mice exhibit a reduced energy expenditure independent of food intake, and a reduced expression of *Ucp1* in both white and brown fat [14, 15]. This phenotype concurs with a reduced BAT activity, and does not match the effects of castration. The AR is widely expressed in a variety of tissues, including the male and female reproductive organs, adipose tissue, liver, skeletal muscle and bone [23]. Tissue-specific knockout models have demonstrated an essential role of the AR for muscle mass and skeletal development [24, 25], and global AR-deficient mice demonstrate growth retardation [26]. Thus, an absence of AR signaling from gestation onwards could substantially affect normal development and therefore body composition, which is not the case for mice that are castrated after maturation.

To more specifically evaluate whether AR expression in BAT could be involved in the effects following castration, we generated BAT-specific AR-deficient mice. Unlike adipose-specific AR-deficient mice that demonstrate a reduced total body weight and decreased gonadal white fat weight [27], BAT-specific AR-deficient mice have a similar body composition as compared to wildtype littermates. Castration of BAT-specific AR-deficient mice promoted metabolic BAT activity as strongly as in wildtype littermates, indicating that castration activates BAT via a mechanism independent of AR signaling. We hypothesized that effects of castration on muscle could underlie this mechanism. The importance of maintaining body temperature for survival has led to the evolution of multiple thermogenic mechanisms within the body. Aside from BAT, muscle is an important site for non-shivering thermogenesis [28]. Muscle-based thermogenesis is upregulated in response to cold in UCP1-deficient mice, whereas a reduced

thermogenic capacity in muscle increases UCP1-dependent thermogenesis in BAT [29]. Since we observed that castration reduces lean mass, which is likely due to muscle wasting [30, 31], we postulate that a reduced thermogenic capacity of muscle could have resulted in a compensatory increase in BAT thermogenesis. This is likely mediated by the sympathetic nervous system, as we measured increased NA levels in BAT from castrated mice. Future experiments that combine castration with sympathetic denervation of BAT or examine the effects of castration at thermoneutrality are necessary to further investigate the importance of sympathetic innervation of BAT in castrated mice.

We conclude that via mechanisms independent of AR signaling in BAT, androgens are potent negative regulators of brown fat activity. We thus postulate that differences in the levels of androgens could be partly responsible for the sex differences in BAT activity. Future research should elucidate the importance of sex hormones for human BAT activity in relation to metabolic health.

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