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Testosterone reduces metabolic brown fat activity in male mice

Marta Lantero Rodriguez1,*, Maaike Schilperoort2,*, Inger Johansson1, Elin Svedlund Eriksson1, Vilborg Palsdottir3,†, Jan Kroon1, Marcus Henriksson1, Sander Kooijman1, Mia Ericson4, Jan Borén1, Claes Ohlsson5, John-Olov Jansson3, Malin C Levin1, Patrick C N Rensen2 and Åsa Tivesten1

1Wallenberg Laboratory for Cardiovascular and Metabolic Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
2Department of Medicine, Division of Endocrinology and Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands
3Department of Physiology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
4Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
5Center for Bone and Arthritis Research, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Correspondence should be addressed to Å Tivesten: asa.tivesten@medic.gu.se

*(M Lantero Rodriguez and M Schilperoort contributed equally to this work)
†(V Palsdottir is now at AstraZeneca, Mölndal, Sweden)

Abstract

Brown adipose tissue (BAT) burns substantial amounts of mainly lipids to produce heat. Some studies indicate that BAT activity and core body temperature are lower in males than females. Here we investigated the role of testosterone and its receptor (the androgen receptor; AR) in metabolic BAT activity in male mice. Castration, which renders mice testosterone deficient, slightly promoted the expression of thermogenic markers in BAT, decreased BAT lipid content, and increased basal lipolysis in isolated brown adipocytes. Further, castration increased the core body temperature. Triglyceride-derived fatty acid uptake, a proxy for metabolic BAT activity in vivo, was strongly increased in BAT from castrated mice (4.5-fold increase vs sham-castrated mice) and testosterone replacement reversed the castration-induced increase in metabolic BAT activity. BAT-specific AR deficiency did not mimic the castration effects in vivo and AR agonist treatment did not diminish the activity of cultured brown adipocytes in vitro, suggesting that androgens do not modulate BAT activity via a direct, AR-mediated pathway. In conclusion, testosterone is a negative regulator of metabolic BAT activity in male mice. Our findings provide new insight into the metabolic actions of testosterone.

Key Words
- brown adipose tissue
- androgens
- androgen receptor
- mice

Introduction

Maintenance of core body temperature is crucial for cellular function and organism survival. Yet, homeostatic body temperature shows certain variation, even within species. For example, body temperature is affected by sex; female mice have an overall higher body temperature than male mice (Sanchez-Alavez et al. 2011). Brown adipose tissue (BAT) is the major site for non-shivering thermogenesis (Morrison 2016), generating heat by oxidation of intracellular lipid stores and uncoupling mitochondrial respiration. Like body temperature, BAT activity may be
affected by sex, and most, but not all, human studies report lower BAT activity as assessed by \(^{[18F]}\)fluorodeoxyglucose uptake in men compared to women (Pfannenberg et al. 2010, Ouellet et al. 2011, van der Lans et al. 2013, Dinas et al. 2015, Fletcher et al. 2020, Becher et al. 2021). In a recent study, adaptive BAT temperature responses were greater in women than in men, irrespective of the stage of the menstrual cycle (Fuller-Jackson et al. 2020).

Sex hormone-mediated effects may underlie sex differences. Testosterone is the most important sex hormone in male mice with more than ten-fold lower levels in females (Nilsson et al. 2015). Castration, which renders male mice testosterone deficient, increases body temperature (Sanchez-Alavez et al. 2011, Hashimoto et al. 2016) supporting that testosterone is involved in the regulation of body temperature. However, relatively little is known about the potential role of androgens in the regulation of BAT activity and existing data are conflicting. Castration has been shown to decrease BAT weight (Movéare-Skritic et al. 2006) and increase BAT \(Ucp1\) mRNA expression (Hashimoto et al. 2016), suggestive of increased BAT activity (Schilperoort et al. 2018). In vitro experiments have indicated direct repressing actions of testosterone on BAT activity; one group found that testosterone treatment of brown adipocytes diminished lipolysis and downregulated thermogenic genes, such as \(Ucp1\) and \(Pgc1a\) (Rodriguez et al. 2002, Monjo et al. 2003, Rodriguez-Cuenca et al. 2007a). \(Ucp1\) expresses the androgen receptor (AR) (Rodriguez-Cuenca et al. 2007b), the receptor for testosterone. AR agonist treatment increases BAT weight in vivo, which may be an indication of reduced BAT activity (Movéare-Skritic et al. 2006). However, global AR deficiency in mice has been shown to decrease the expression of \(Ucp1\) in BAT (Fan et al. 2005, Yanase et al. 2008). It is not known whether androgens modulate BAT activity in vivo and through which pathway(s) such an effect could be mediated.

In the current study, we aimed to clarify the role of testosterone in metabolic BAT activity. Further, we investigated potential pathways involved and addressed the question of whether the effects of castration can be mimicked by BAT-specific AR deficiency.

Material and methods

Animals

Male C57Bl/6J mice were purchased from Charles River. Male BAT-specific ARKO mice were generated by breeding \(AR^{+/\text{lox}}\) female mice (from Dr Verhoeven, Katholieke Universiteit Leuven, Belgium) (De Gendt et al. 2004) with male \(Ucp1\)-Cre mice (B6.FVB-Tg(Ucp1-cre)1Evdr/J; Jackson Laboratories). Male mice with the genotype \(AR^{\text{lox}/\text{lox}}\text{-}\text{Ucp1-Cre}^{+/\text{cre}}\) are BAT-AR KO; littermate controls are \(AR^{\text{lox}/\text{lox}}\text{-Ucp1-Cre}^{+/\text{cre}}\). Littermate controls were used in all experiments. The mice were housed in a temperature-controlled room with a 07:00–19:00 h light cycle and consumed a soy-free chow diet (Cat# 2019 Teklad global 19% protein extruded rodent diets, Harlan Laboratories) and tap water ad libitum. All experiments followed the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Care and Use in Gothenburg, and the Leiden University Ethical Review Board.

Castration (orchiectomy)

Wild-type, BAT-AR KO, and control mice were anesthetized with isoflurane (IsoFlo\textsuperscript{®} vet., Vnr 002185, Zoetis) and either sham-operated or bilaterally castrated/orchiectomized (ORX) at 10–11 weeks of age unless otherwise specified. Buprenorphine (Temgesic\textsuperscript{®}, RB Pharmaceuticals Ltd) was used for analgesia after all surgical procedures.

Castration and testosterone replacement

Tissues were collected from mice 3 weeks after the bilateral castration or sham operation. Following the surgery, mice were subcutaneously injected at the end of the day with vehicle (pure corn oil, Cat# C8267, Sigma) or testosterone propionate (Cat#86541, Sigma) every 3 days for 3 weeks. A working solution of testosterone (2.5–3 mg/kg/day for a 25 g mouse) was made by dissolving 45 mg of testosterone propionate in 0.4 mL of 100% ethanol and then adding 30 mL of pure corn oil.

Uptake of radiolabeled triglyceride-derived fatty acids

Glycerol tri\([^{3}H]\)oleate-labeled triglyceride (TG)-rich lipoprotein-like emulsion particles (80 nm) were prepared and characterized as described previously (Rensen et al. 1995). Mice were fasted for 4–6 h and intravenously injected with 200 \(\mu\)L of emulsion particles (1 mg TG per mouse) via the tail vein or retro-orbitally, 2 h before the 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[3H]oleate-derived radioactivity was quantified and expressed per organ or per gram of wet tissue weight.

**Telemetry and indirect calorimetry**

Telemetry devices (G2 E-mitter, MiniMitter, Bend, OR, USA) were implanted via a 1 cm incision through the skin and peritoneum. Following implantation, the peritoneum was stitched with Polysorb 5-0 suture (Covidien, Dublin, Ireland), and the skin was closed with Reflex 7 clips (CellPoint Scientific, Inc., Gaithersburg, MD, USA). The surgery was performed under 3% isoflurane (Baxter, Deerfield, IL, USA) anesthesia, and buprenorphine (Temgesic®, RB Pharmaceuticals Ltd) was administered subcutaneously for analgesia. The mice were left to recover for 1 week before the start of experiments. Oxygen consumption (VO$_2$) was measured by indirect calorimetry in an INCA metabolic system (Somedic, Hörby, Sweden) as previously described (Wernstedt *et al.* 2006). The system comprises a sealed chamber with regulated airflow and temperature. It also includes sensors for the MiniMitter telemetry system, which are designed to measure core body temperature with 0.01°C accuracy and three-dimensional locomotor activities via the implanted G2 E-mitter transponders. Data were collected every minute for all variables except for VO$_2$, which was measured every second minute, over 21 h at 20°C.

**DEXA**

Measurements of body composition, fat, and lean mass were performed by dual-energy x-ray absorptiometry (DEXA) using the Lunar PIXImus mouse densitometer (Wipro GE Healthcare).

**Tissue collection**

Mice were anesthetized, blood was drawn from the left ventricle, and the mice were perfused with saline or PBS under physiological pressure. Interscapular BAT (iBAT) and subscapular BAT (sBAT) were dissected, snap-frozen, and stored at −80°C. Interscapular BAT was also embedded in OCT and frozen slowly for histological analysis.

**Histological analysis**

Interscapular BAT was cut into serial 10 µm sections and was stained with hematoxylin (Cat# 1820, Histolab) and eosin (Cat# ACRO409430250, VWR) using standard protocols. The area of intracellular lipid vacuoles was quantified by a blind observer using Visiopharm Integrator System (Version 5.3.0.1562, Visiopharm). Immunofluorescence staining of UCP1 was performed by using rabbit anti-mouse UCP1 (polyclonal, Cat#ab23841, Abcam, 1 µg/mL), followed by a secondary antibody AF595-conjugated donkey anti-rabbit IgG (Cat# 711-587-003, Jackson Immuno Research Laboratories; 1 µg/mL) and staining with DAPI (Cat# D9542 Sigma-Aldrich).

**Triglyceride analysis of BAT**

Lipids within one lobe of interscapular BAT were extracted using the BUME method (Lofgren *et al.* 2016). Triglycerides were quantified by direct infusion (shotgun) analysis on a QTRAP 5500 mass spectrometer (Sciex, Concord, Canada) equipped with a robotic nanoflow ion source, TriVersa NanoMate (Advion BioSciences, Ithaca, NJ). The analysis was performed in positive ion mode by neutral loss detection of 11 common acyl fragments formed during a collision-induced dissociation according to previous work (Murphy *et al.* 2007). Glycerol-d$_4$-hexadecanoate (CDN isotopes, Quebec, Canada) was added during the extraction and used for quantification.

**Noradrenaline content in BAT**

The NA content of a lobe of interscapular BAT was determined by HPLC followed by electrochemical detection. The tissue was kept cold and homogenized for 3 × 20 s in 500 µL homogenization solution (5 mM EDTA in 0.1 M perchloric 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 (100%) 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, Västra Frölunda, Sweden) and diluted in milliQ water (100×). NA was analyzed on a reversed phase column (Capsel Pak’ 50 × 2 mm, 3 µm C18; Phenomenex, Værløse, Denmark, operated at 30°C) with a mobile phase (flow rate 0.3 mL/min) consisting of 150 mM NaH$_2$PO$_4$, 4.76 mM citric acid, 3 mM sodium dodecyl sulfate, 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 vs the cell. An external standard containing 2.95 fmol/µL of noradrenaline was used to identify and quantify NA content in the sample (Chromeleon software, Thermo Fisher Scientific).
Serum levels of testosterone were measured by gas chromatography–tandem mass spectrometry (GC–MS/MS), as previously described (Nilsson et al. 2015). Briefly, after the addition of isotope-labeled standards, steroids were extracted to chlorobutane, purified on a silica column, and derivatized using pentafluorobenzylhydroxylamine hydrochloride followed by pentafluorobenzoyl chloride. Steroids were analyzed in multiple reactions monitoring mode with ammonia as reagent gas using an Agilent 7000 triple quadrupole mass spectrometer equipped with a chemical ionization source. Assay performance, including intra-assay and inter-assay coefficients of variations (CVs), has been published previously (Nilsson et al. 2015). The lower limit of quantification (LOQ) of the assay is 8 pg/mL and the lower limit of detection (LOD) is 4 pg/mL for testosterone. In 17-week-old male mice that were castrated or sham-castrated at 13 weeks of age, all sham-castrated mice and 5/7 castrated mice had serum testosterone above LOD of the assay. In the castrated group, two mice had undetectable levels; these values were set to LOD of the assay.

RNA isolation and real-time RT-PCR

Total RNA was extracted from BAT with RNeasy Plus Universal Mini Kit (Cat#73404; Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from total RNA with a high-capacity cDNA RT kit (Cat#4374966; Applied Biosystems). RT-PCR analysis was done with predesigned TaqMan Gene Expression Assays (Applied Biosystems): Ucp1 (Mm01244861_m1), Pgc1a (Ppargc1a; Mm01208835_m1) Elov3 (Mm00468164_m1), Lpl (Mm00434764_m1), C/EBPα (Mm_01135198_m1), Hsl (lipase; Mm00495359_m1), Atgl (Pnpla2; Mm00503040_m1), Plin5 (Mm00508852_m1), Cidea (Mm00432554_m1), Ppara (Ppara_Mm00627559_m1), Pparg (Mm01184322), Ar (Mm00426888_m1), and 18s (Hs99999901_s1) as a reference gene. The analyses were run in Viia 7 Real-time PCR System (Applied Biosystems). Data were normalized to the reference gene 18s, and gene expression was calculated with the 2−ΔΔCt method.

DNA quantification

Genomic DNA from interscapular and subscapular BAT, inguinal and mesenteric white adipose tissue, kidney, muscle, and spleen was isolated with DNeasy blood and tissue kit (Cat#69504; Qiagen) according to the manufacturer’s instructions. Amplification of genomic DNA was detected with SyBR green master mix (Cat#4367659; Applied Biosystems) and Viia 7 Real-time PCR System (Applied Biosystems). The following primer pairs were used: Ar exon 2: forward GGACCATGTTTTACCCATCG and reverse CCACAAGTGAGCCTCGTA; Ar exon 3: forward TCTATGTCAGCAAGAACG and reverse CCCAGATCATCCCTGCTT. Ct values for Ar exon 2 were normalized to Ct values for Ar exon 3 by the 2−ΔΔCt method.

Cell culture and experiments

Immortalized preadipocytes were cultured as previously described (Kroon et al. 2018). Details of the generation (achieved by doxycycline-controlled expression of simian virus 40 large tumor antigen with a repressor-based Tet-On system) and brown adipocyte properties of the immortalized brown adipocyte cell line (clone #6) have been published previously (Liu et al. 2019). Briefly, preadipocytes were differentiated for 14 days. During the last 3 days of differentiation, cells were grown in a medium containing hormone-deprived (charcoal-stripped) serum. Cells were treated with DHT (10 nM) or vehicle for 8 h, followed by stimulation with NA (1 µM) or vehicle for 16 h. Hereafter, medium was collected to measure glycerol concentrations using a commercially available assay (Instruchemie, Delfzijl, The Netherlands). Cells were differentiated in 96-well Seahorse Bioscience assay plates for measurement of the oxygen consumption ratio (OCR) using the Seahorse Bioscience XF96 extracellular flux Analyzer (Seahorse Bioscience). After differentiation, cells were pre-treated with DHT (10 nM) or vehicle in Agilent Seahorse XF Base Medium supplemented with 10% hormone-deprived serum and 25 mM glucose (pH of final medium adjusted to 7.4) for 8 h before starting Seahorse analysis. NA or vehicle diluted in Seahorse medium was pre-loaded 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.

Isolation of mature brown adipocytes and glycerol release quantification

Interscapular BAT (approx. 100 mg) was minced to 0.5–1 mm3 pieces using scissors. The tissue was digested in 1 mL digestion medium (1 mg/mL collagenase B, Worthington, 1 mg/mL soybean trypsin inhibitor, Worthington and 4% fatty acid-free BSA, Sigma, in Hank’s balanced solution, Gibco) for 30 min at 37°C with gentle mixing (300 rpm). Every 10 min, the tissue solution was
gently mixed by pipetting. The first two times the tip was cut a bit to make the bigger pieces pass. After 30 min, no tissue pieces were visible, and the cells were washed three times in modified Krebs–Ringer Bicarbonate buffer (KRBB) (15 mM NaHCO₃, 1 mM CaCl₂, 20 mM HEPES, 4% fatty acid-free BSA, 100 U/mL penicillin and 100 µg/mL streptomycin in KRBB, Sigma). Floating mature adipocytes were washed by centrifugation at low speed (270 g) for 5 min, the infranatant was gently removed by a syringe and the cells were left at room temperature for 30 min. Cells from sham and ORX operated mice were pooled separately and live cells were counted in a NucleoCounter (Chemometec). Cells (3 × 10⁶) were seeded in 300 µL in modified Krebs solution in duplicates and harvested after 3, 6, and 9 h by transferring 125 µL cell suspension to 0.45 µm centrifugal filter units (Millipore). Units were centrifuged at 10,000 g for 30 s, and free glycerol concentration in the supernatant was measured using the Glycerol Assay kit (Sigma MAK117) according to the manufacturer’s protocol.

Statistics

Statistical evaluations were performed with Prism software (GraphPad Software, Inc., version 8.2.1). All variables were tested for normal distribution by Shapiro–Wilk normality test. For variables that passed the normality test with or without log transformation, two-group comparisons were performed by two-tailed unpaired t-test, three-group comparison with one-way ANOVA followed by Dunnett’s post hoc test, and four-group comparison by two-way ANOVA. OCR data were analyzed by two-way ANOVA with repeated measures. Data that did not pass normality tests were analyzed using Mann–Whitney U-test (two groups) or Kruskal–Wallis test followed by Dunn’s post hoc test (three groups). P values < 0.05 were considered statistically significant. Unless otherwise specified, results are represented as mean ± S.E.M.

Results

Castration reduces weight and lipid content of BAT

Castration of male mice reduced mean testosterone levels by 94% (Fig. 1A). We found that castration reduced BAT weight already 6 days after surgery of adult mice, and at 21 days, there was a 34% reduction in iBAT weight (Fig. 1B), with a similar effect on iBAT weight adjusted to body weight (Fig. 1C). The effect on BAT weight was maintained long-term (Fig. 1D). Triglyceride (TG) content of the tissue was also reduced 6 days after castration of adult mice (Fig. 1E). Analysis of the fatty acid (FA) composition of TG showed that castration reduced the relative content of palmitic acid (C16:0) and increased the relative content of the generally less abundant longer chain FAs, such as oleic acid (C18:1), dihomo-γ-linoleic acid (20:3), arachidonic acid (C20:4), and docosahexaenoic acid (C22:6) (Fig. 1F), which may be secondary or consistent with the FA elongation that occurs during BAT activation (Townsend and Tseng 2014). Histological analysis showed that castration reduced the lipid droplet content (Fig. 1G and H) and seemed to increase the intensity of UCP1 staining in BAT (Fig. 1I). Further, castration resulted in a modest upregulation of mRNA expression of genes involved in lipid metabolism (Lpl, Elovl3) and thermogenesis (Pgc1a, Ucp1) in BAT (Fig. 1J). Mature brown adipocytes isolated from castrated mice released significantly more glycerol than brown adipocytes from sham-operated mice (Fig. 1K), supporting increased basal lipolysis in castrated mice. Overall, these results show that testosterone deficiency reduces BAT weight and lipid content and modifies the gene expression of BAT.

Castration increases, and testosterone replacement reduces, fatty acid uptake by BAT

Prolonged BAT activation leads to increased FA uptake to replenish the lipid droplets and secure access to fuel (Hoeke et al. 2016). As castration reduced lipid droplet content and tissue weight of BAT, we hypothesized that castration increases lipid uptake by BAT and that testosterone replacement to castrated mice prevents this effect. To test this hypothesis, we determined the tissue-specific uptake of TG-derived FAs in sham-operated and castrated mice treated with vehicle or testosterone. We showed that testosterone replacement reversed the reduction in BAT weight caused by castration (Fig. 2A). A relatively large percentage of the given dose of TG-derived FAs was taken up by the BAT depots, and FA uptake of BAT was markedly enhanced by castration but returned to levels seen in sham-operated mice upon testosterone replacement (Fig. 2B). Expressed per gram of tissue, the relative FA uptake was higher in BAT compared to other organs (Fig. 2C).
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Figure 1
Castration reduces weight and lipid content of BAT. (A) Serum testosterone levels in male mice that were sham-operated (n = 8) or castrated (ORX, n = 7) at 13 weeks of age. Serum was collected 4 weeks after surgery. (B) Interscapular BAT (iBAT) weight in mice that were sham-operated (n = 4–9) or castrated (ORX, n = 5–9) at 11–14 weeks of age. iBAT was collected at 3, 6, and 21 days after surgery. (C) iBAT weight relative to body weight 21 days after surgery in sham-operated and ORX mice. (D) iBAT weight in mice that were sham-operated (n = 14) or ORX (n = 15) at 3 weeks of age. iBAT was collected at 35 weeks of age. (E) iBAT triglyceride (TG) content in mice that were sham-operated (n = 4–5) or ORX (n = 4–5) at 12–14 weeks of age. iBAT was collected at 3, 6, and 12 days after surgery. (F) iBAT fatty acid (FA) composition of TG at 12 days following surgery of 14-week-old mice (n = 4 per group). (G, H, I, J and K) Mice were sham-operated (n = 6) or ORX (n = 6) at 11 weeks of age, and iBAT was collected 21 days after surgery. (G and H) Representative images of hematoxylin-eosin stained sections of iBAT and quantification of lipid content. Scale bar, 100 µm. (I) Representative images of UCP1-staining of iBAT. Scale bar, 100 µm. (J) Expression of genes involved in BAT activity, calculated relative to 18S (Ct mean ± s.e.m. for 18S was 7.82 ± 0.05 in ORX and 7.90 ± 0.07 in sham). (K) Glycerol release from isolated brown adipocytes, results are from three separate experiments in duplicate. Glycerol release in sham-castrated mice was 302 ± 63, 180 ± 36, and 133 ± 27 pmol/min/10^6 cells after 3, 6, and 9 h incubation, respectively. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the sham group (two-tailed unpaired Student's t-test). Bars indicate means, error bars indicate s.e.m. DGLA, dihomo-γ-linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.
Castration-induced increase in metabolic BAT activity is independent of AR in BAT

AR activation by the natural AR agonist dihydrotestosterone (DHT) has been shown to reverse the decrease in BAT weight after castration (Movère-Skrtic et al. 2006). Therefore, we next investigated whether the castration-induced increase in BAT activity is explained by a reduction in AR signaling in brown adipocytes. Treatment of murine immortalized brown adipocytes with DHT did not affect the basal or noradrenaline (NA)-induced respiration (Fig. 3A) or glycerol release (Fig. 3B), indicating that AR signaling does not have a direct effect on brown adipocytes in vitro. To address the role of AR signaling in BAT function in an in vivo setting, we generated male mice with an AR deletion in brown adipocytes (BAT-ARKO) using an Ucp1-Cre construct. We observed a 40% reduction of Ar exon 2 DNA in total interscapular as well as subscapular BAT DNA (Fig. 3C), as expected from the around 50% adipocyte content of fat depots (Kang et al. 2014). This reduction is in the range of other studies using the Ucp1-Cre construct for targeting brown adipocytes in BAT (Turpin et al. 2014).

Further, Ar DNA was reduced by 11% in inguinal (brite) fat, but no deletion was detected in mesenteric (white) fat, muscle, kidney, or spleen (Fig. 3C). mRNA expression of Ar in BAT was reduced by 46% while Ucp1 mRNA levels were unchanged (Fig. 3D). BAT weight was not different between BAT-ARKO and Ucp1-Cre+/- mice and castration similarly reduced BAT weight in both genotypes (Fig. 3E). FA uptake per gram BAT (Fig. 3F) or whole BAT (Fig. 3G) was not affected by genotype and similarly increased in both BAT-ARKO and Ucp1-Cre+/- mice by castration. These results show that the castration-induced increase in metabolic BAT activity is not mimicked by AR depletion in BAT.

Castration, but not AR-knockout in BAT, increases body temperature mainly at ZT 8–12

We next studied whether body temperature changes by castration follows a circadian rhythm. Mice were kept under a standard 12 h light: 12 h darkness cycle and time of the day was denoted as zeitgeber time (ZT), where ZT
Figure 3
Castration-induced increase in metabolic BAT activity is not dependent on AR in BAT. (A and B) Immortalized brown adipocytes were treated with vehicle or DHT (10 nM) for 8 h. (A) Oxygen consumption rate (OCR) before and after stimulation with NA (1 µM) or vehicle (n = 19–22 wells per group). (B) Glycerol release as % of vehicle before and after stimulation with NA (1 µM) or vehicle for 16 h (n = 3 per group). Data were analyzed by two-way repeated-measures ANOVA. Bars indicate means, error bars indicate s.e.m. (C and D) At 12 weeks of age, BAT-ARKO (n = 11), Ucp1-Cre+/- mice (n = 8) were sacrificed and organs were collected. (C) Relative Ar exon 2 DNA from various organs, including interscapular BAT (iBAT), subscapular BAT (sBAT), inguinal WAT (IngWAT), mesenteric WAT (MesWAT), and extensor digitorium longus (EDL) muscle. (D) Ar and Ucp1 mRNA expression in interscapular BAT (iBAT). **p < 0.01, ****p < 0.0001 compared to the Ucp1-Cre+/- group (two-tailed unpaired Student’s t-test). Bars indicate means, error bars indicate s.e.m. (E, F and G) At 10 weeks of age, Ucp1-Cre+/- and BAT-ARKO male mice were castrated (ORX) or sham-operated and at 24 weeks of age tri[3H]oleate-derived radioactivity in BAT was determined (n = 9–10/group). (E) Organ weights. (F and G) Uptake of glycerol tri[3H]oleate-derived radioactivity per gram of wet tissue or whole organ. Data were analyzed by two-way ANOVA. Bars indicate means, error bars indicate s.e.m.
0 and ZT 12 corresponds to the start of the light and the dark period, respectively. We specifically addressed time periods with low BAT activity/high locomotor activity (ZT 4–6), high BAT activity/low locomotor activity (ZT 8–12), and high BAT activity/high locomotor activity (ZT 12–18) (van den Berg et al. 2018). Castration, but not AR deficiency in BAT, increased body temperature at ZT 8–12, and the mean body temperature over the 21 h period was higher in castrated mice (Fig. 4A). During ZT 8–12, but not across 21 h, locomotor activity was increased by castration (Fig. 4B). Unadjusted oxygen consumption was reduced by castration (Fig. 4C). In the same mice, castration reduced body weight (Fig. 4D) and lean mass (Fig. 4E) but had no statistically significant effect on fat mass (Fig. 4F). Castration had a minor effect on oxygen consumption adjusted to lean mass, which was increased during ZT 8–12 only (Fig. 4G). The respiratory exchange ratio was similar across the groups (Fig. 4H). Overall, these results demonstrate that there is an effect of castration but not BAT AR deficiency on body temperature and that the effect of castration on body temperature shows a diurnal pattern.

**Castration slightly increases noradrenaline content in BAT**

As we did not find evidence for a direct effect of androgens/AR on BAT activity, we next asked whether the regulation of BAT activity by androgens might involve central thermoregulatory pathways. Since the sympathetic nervous system is the most important regulator of BAT thermogenic activity (Kooijman et al. 2015), we hypothesized that castration increases NA content in BAT. Indeed, NA concentration in BAT was slightly increased within 6 days following castration (Fig. 5A). This effect was maintained for at least 21 days after surgery and was present even without correction for the weight of the lobe (Fig. 5B).

**Discussion**

Here we investigated the role of androgens in BAT activity in male mice. We demonstrated that androgen depletion by castration increases metabolic BAT activity. Mechanistically, the link between androgens and metabolic BAT activity is independent of AR in BAT.

In the present study, we used lipid tracers to show that castration increased lipid uptake by BAT in vivo, which is an indirect measure of an increased metabolic BAT activity (Hoek et al. 2016, Schilperoort et al. 2016).

Compared to glucose uptake, lipid uptake is a better measure of BAT metabolic activity, as activated BAT selectively increases oxidation of lipids over glucose and replenishes intracellular lipid stores by taking up triglyceride-derived FA (Ouellet et al. 2012, Schilperoort et al. 2016, Carpenter et al. 2018). It should be noted that a high increase in FA uptake may even be underestimated given that rapid combustion of [3H]oleate by BAT will result in the release of radiolabel from BAT in the form of H2O. As lipoprotein lipase is important for this uptake (Bartelt et al. 2011), the large increase in FA uptake seems incongruent with the minor regulation of Lpl mRNA by castration found here. However, lipoprotein lipase activity is also regulated post-transcriptionally and post-translationally (Basu & Bornfeldt 2020). Further, other yet unidentified factors may mediate the increased FA uptake. The increased FA uptake after castration is in line with our other observations (i.e. lower BAT weight, reduced lipid content in BAT, increased expression of several lipolytic and thermogenic genes in BAT, and increased basal lipolysis of isolated brown adipocytes) that suggest increased BAT activity, although BAT thermogenic activity has not been directly assessed in our study. While we found a modest increase in Ucp1 mRNA levels by castration, UCP1 activity is predominantly regulated through post-transcriptional and post-translational mechanisms (Cannon & Nedergaard 2004, Takahashi et al. 2015), which have not been studied here. Our finding that castration reduces BAT weight and increases Ucp1 mRNA expression in BAT is consistent with previous observations (Movérare-Skrtic et al. 2006, Hashimoto et al. 2016).

Previous in vitro studies suggest that androgen/AR signaling in BAT directly impairs brown adipocyte activity. Concentrations of testosterone as low as 1 nM have been reported to decrease lipolysis (estimated by glycerol release) and expression of Pgc1a, an important regulator of mitochondrial biogenesis and thermogenic function, in murine brown adipocytes (Monjo et al. 2003, Rodriguez-Cuenca et al. 2007a). Treatment of brown adipocytes with high doses (100 nM) of testosterone also reduced expression of Ucp1, an effect that was blocked by the AR antagonist flutamide (Rodriguez et al. 2002). Here we could not replicate the effects on glycerol release using the natural AR agonist DHT, which is about 2- to 3-fold more potent than testosterone (Wright et al. 1996). Also, we did not find any effect of 10 nM DHT on cellular respiration, a direct measure of brown adipocyte activity. Consistent with our in vitro data, we found that BAT-specific AR deficiency had no effects on BAT weight, TG-derived FA uptake by BAT, or body temperature. Of note, our BAT-ARKO mice

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Castration, but not AR-knockout in BAT, increases body temperature mainly at ZT 8–12. (A, B, C, D, E and F) At 11 weeks of age, Ucp1-Cre<sup>+/−</sup> and BAT-AR<sup>−/−</sup> male mice were ORX (Ucp1-Cre<sup>+/−</sup> n = 8, BAT-AR<sup>−/−</sup> n = 6) or sham-operated (Ucp1-Cre<sup>+/−</sup> n = 9, BAT-AR<sup>−/−</sup> n = 6) and at 14 weeks of age body composition was measured by DEXA and at 15–16 weeks indirect calorimetry analysis performed. Times are given as zeitgeber times (ZTs), with ZT0 at the onset of the light period and ZT12 at the onset of the dark period. (A, B, C, G and H) Representative profile of the different parameters (core body temperature, locomotor activity (LMA), oxygen consumption (VO<sub>2</sub>), and respiratory exchange ratio (RER)) measured over a 21-h period at 20°C and mean values analyzed at different time intervals during the day (ZT 4–6, ZT 8–12 and ZT 12–18) and whole 21 h study period (ZT 4–1). (A) Core body temperature, (B) locomotor activity (LMA), (C) oxygen consumption (unadjusted) (VO<sub>2</sub>), (D) body weight at 14 weeks of age (E), lean mass, (F) fat mass, (G) oxygen consumption adjusted to lean mass and (H) respiratory exchange ratio (RER). Data were analyzed by two-way ANOVA. Bars indicate means, error bars indicate s.e.m.
disorders or treatments have increased body temperature; simple association studies between these variables will likely be confounded by disorders such as obesity (Kahn & Brannigan 2017). Interestingly, both androgen withdrawal (androgen deprivation therapy of prostate cancer) in men (Jones et al. 2012, Gonzalez et al. 2018) and estrogen withdrawal (menopause) in women (Freedman 2014) have been associated with the occurrence of hot flashes. In menopausal women, hot flashes seem to be triggered by small elevations in core body temperature within a narrowed thermoneutral zone (Freedman 2014), while to our knowledge there are no similar studies performed in men with testosterone deficiency.

In mass spectrometry-based analyses performed by our group (shown here and in Nilsson et al. 2015), testosterone levels were around 25–50 pg/mL in female mice, as compared to 225–8000 pg/mL in gonadal-intact male mice (showing a large biological variation), and 4–25 pg/mL in castrated male mice. Thus, it is conceivable that testosterone-mediated regulation of BAT activity and body temperature may contribute to sex differences in these variables. Notably, while female mice have an overall higher body temperature than male mice (Sanchez-Alavez et al. 2011), the sex difference in BAT activity in rodents is less established. Further, there is a paucity of studies directly comparing body temperature or BAT activity in castrated male rodents with gonadal-intact females, which also display variation in body temperature across the ovarian cycle (Sanchez-Alavez et al. 2011). BAT from male rats reportedly exhibits a lower Ucp1 expression and mitochondrial respiration as compared to BAT from gonadal-intact females (Rodriguez-Cuenca et al. 2002), although the results of that study may be confounded by body weight differences between male and female rats. Sex differences in BAT activity in humans are supported by most, but not all, studies (Pfannenberg et al. 2010, Ouellet et al. 2011, van der Lans et al. 2013, Dinas et al. 2015, Fletcher et al. 2020, Becher et al. 2021). In a recent study, adaptive BAT temperature responses were greater in women than in men, irrespective of cycle stage (Fuller-Jackson et al. 2020). However, during the follicular phase only, the increase in BAT temperature during cold exposure was lower in women vs men (Fuller-Jackson et al. 2020). These data support that factor(s) that vary during the ovarian cycle are important regulators of BAT activity in women, requiring awareness in studies of the sexual dimorphism in BAT physiology.

In the present study, we found that castration slightly increased NA content in BAT. This finding may support that castration increases the sympathetic outflow to
BAT, as sympathetic activation following cold-induced thermogenesis causes increased NA release to BAT (Blaszkiewicz et al. 2019). As NA from sympathetic nerve endings activates murine β2-adrenergic receptors in BAT, leading to increased lipolysis, FA uptake from blood, and heat production by the mitochondria (Hoeke et al. 2016), an increased NA content in BAT is in line with BAT activation. Importantly, other mechanisms for increased tissue NA content, such as altered NA reuptake or turnover, cannot be excluded. Of note, testosterone generally increases sympathetic nervous signaling (Dibner & Black 1976, Keast 2000, Ermis et al. 2010), but it is plausible that androgens modulate sympathetic output in a tissue-specific manner. Future experiments should address the involvement of central thermoregulatory pathways, which may be mediated by hypothalamic, medullary, and/or spinal sites (Nakamura & Nakamura 2018).

The effects of castration on metabolic BAT activity and body temperature shown in the present study may be associated or independent of each other. Notably, thermogenic BAT activity has not been studied here. In line with other studies showing castration-induced muscle wasting (Jiao et al. 2009, Serra et al. 2013), we confirmed that castration reduces lean mass in male mice. Skeletal muscle is an important site for thermogenesis (Periasamy et al. 2017) and loss of muscle mass reduces its thermogenic contribution to body temperature. Therefore, it is possible that castration increases BAT activity as a compensatory mechanism. It has been reported that reduced thermogenic capacity in muscle increases UCP1-dependent thermogenesis in BAT (Bal et al. 2017). Interestingly, we found that locomotor activity as well as body temperature was increased by castration at ZT 8–12. During the same time frame, we found increased oxygen consumption, which may be related to both of these findings. However, the effect on oxygen consumption was minor and only appeared after normalization for lean mass. Further research is needed to unravel the physiological relevance of these findings.

In conclusion, we show that testosterone is a negative regulator of metabolic BAT activity in male mice. This finding provides new insight into the metabolic actions of androgens, which may have important clinical implications. To date, there are no clinical studies examining the role of androgens on BAT activity. Our findings raise the question of whether androgen deficiency may modulate responses to BAT-directed therapeutic interventions, an area of intense ongoing research. Future research efforts should address the importance of androgens for BAT activity in humans.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

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