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Castration of Male Mice Induces Metabolic Remodeling of the Heart

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Abstract

Androgen deprivation therapy of prostate cancer, which suppresses serum testosterone to castrate levels, is associated with increased risk of heart failure. Here we tested the hypothesis that castration alters cardiac energy substrate uptake, which is tightly coupled to the regulation of cardiac structure and function. Short-term (3-4 weeks) surgical castration of male mice reduced the relative heart weight. While castration did not affect cardiac function in unstressed conditions, we observed reductions in heart rate, stroke volume, cardiac output, and cardiac index during pharmacological stress with dobutamine in castrated vs sham-operated mice. Experiments using radiolabeled lipoproteins and glucose showed that castration shifted energy substrate uptake in the heart from lipids toward glucose, while testosterone replacement had the opposite effect. There was increased expression of fetal genes in the heart of castrated mice, including a strong increase in messenger RNA and protein levels of β-myosin heavy chain (MHC), the fetal isoform of MHC. In conclusion, castration of male mice induces metabolic remodeling and expression of the fetal gene program in the heart, in association with a reduced cardiac performance during pharmacological stress. These findings may be relevant for the selection of treatment strategies for heart failure in the setting of testosterone deficiency.

Key Words: castration, mice, heart, metabolism

Abbreviations: βMHC, β-myosin heavy chain; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; BSA, bovine serum albumin; LV, left ventricular; mRNA, messenger RNA; ORX, orchiectomized; Sham, sham-castrated.

Testosterone, which is the main bioactive sex steroid in men, exerts important physiological effects on the heart. Studies show a direct hypertrophic effect of testosterone on the myocardium, and the androgen receptor in cardiomyocytes may mediate such effects [1]. The pubertal increase in heart weight in relation to body weight is larger in men compared to women and testosterone is suggested to play a role in this sex difference [1]. Androgen deprivation therapy of prostate cancer, which suppresses serum testosterone to castrate levels, is associated with increased risk of heart failure [2–4], supporting the notion that testosterone has an important physiological role in maintaining cardiac function in men. Understanding the mechanisms underlying cardiac dysfunction in testosterone deficiency is important for the selection of appropriate treatment strategies.

The heart possesses great metabolic flexibility and uses a mixture of energy-providing substrates, most importantly fatty acids ($\sim 60\%$) and glucose ($\sim 30\%$) [5]. In most settings, cardiac stress is associated with changes of cardiac metabolism and substrate preference, often toward increased glucose utilization. Further, long-term increases in glucose supply and utilization are associated with a shift to a fetal transcriptional program in cardiomyocytes, so that fetal genes are reexpressed in the adult heart [5]. While this transition initially may support cardiac structure and function, it also reduces cardiac efficiency and may contribute to the pathogenesis of heart failure [6]. To date, relatively few studies have addressed the cardiac response to testosterone deficiency in male rodents [7–9] and, to our knowledge, there are currently no in vivo data on potential cardiac metabolic remodeling in testosterone deficiency.

In the present study, we tested the hypothesis that surgical castration, which reduces testosterone levels by approximately 95% in male mice [10], alters cardiac energy substrate uptake. We assessed cardiac function during rest and pharmacological stress by echocardiography, measured triglyceride-derived fatty acid and glucose uptake in the heart using radiolabeled tracers, and analyzed the expression of genes associated with activation of the fetal metabolic program in the heart.

Materials and Methods

Animals

Male C57Bl/6J mice (Charles River), aged 9 to 14 weeks, were used in all experiments. The mice were housed in a temperature- and humidity-controlled room with a 06:00 to 18:00 hours light cycle and consumed a soy-free chow diet (catalog No. 2019 Teklad Global 19% protein extruded rodent diets, Harlan Laboratories) and tap water ad libitum. All animal experiments were conducted in compliance with local guidelines, and the Ethics Committee on Animal Care and Use in Gothenburg or the Leiden University Ethical Review Board approved all procedures.

Castration (Orchidectomy) and Testosterone Replacement

Mice were anesthetized with isoflurane (IsoFlo vet, Vnr 002185, Zoetis) and either bilaterally castrated (orchiectomized; ORX) or sham-castrated (Sham). Buprenorphine (Temgesic, RB Pharmaceuticals Ltd) was used for analgesia after all surgical procedures. Following castration, some mice were subcutaneously injected with vehicle (pure corn oil, catalog No. C8267, Sigma) or a physiological dose [11] of testosterone propionate (3 mg/kg/day; catalog No. 86541, Sigma) every 3 days for 3 weeks. A working solution of testosterone 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. Success of castration and testosterone replacement was verified by recording the weight of the androgen-sensitive seminal vesicles at the study end.

Echocardiography

Echocardiographic assessment of cardiac function [12] was performed using a VEVO 770 system (VisualSonics). The system includes an integrated rail system for consistent positioning of the ultrasound probe. Hair removal cream was applied to the chest before the examination to minimize resistance to ultrasonic beam transmission. The animals were anesthetized with isoflurane (1.1%) and placed on a heating pad with paws connected to electrocardiographic electrodes. A 45-MHz linear transducer (RMV 704) was used. An optimal parasternal long-axis projection (ie, visualization of the aortic and mitral valve and the maximum distance between the aortic valve and the cardiac apex) was acquired using the electrocardiographic-gated kilohertz technique. The probe was then rotated 90° and a parasternal short-axis cine loop of more than 1000 frames per second was acquired at 1, 3, and 5 mm below the mitral annulus. When the baseline examination was performed the probe was positioned in parasternal long-axis projection and dobutamine (2 µg/g body weight) was injected intraperitoneally. Seven minutes post injection, at the time point of maximum effect, echocardiographic examination was performed once again using the same protocol. Mice (4 ORX, 4 Sham) were excluded from the study because their heart rate increased less than 100 beats/min [13, 14]. The stored data were evaluated offline using VevoLab software system (VisualSonics) in a blinded fashion. End-diastolic and end-systolic left ventricular (LV) volumes and ejection fraction were calculated with the biplane Simpson formula using the 3 parasternal short-axis views and the parasternal long-axis view. End-diastole was defined at the onset of the QRS complex, and end-systole was defined as the time of peak inward motion of the interventricular septum. At least 3 beats were averaged for each measurement.

Uptake of Radiolabeled Triglyceride-derived Fatty Acids and Glucose

Glycerol tri[^{3}H]oleate-labeled triglyceride-rich lipoprotein-like emulsion particles (80 nm) were prepared and characterized as described previously [15] and [^{14}C]deoxyglucose was added (ratio ^{3}H:^{14}C = 4:1). Mice were fasted for 4 to 6 hours and intravenously injected with 200 µL of emulsion particles (1 mg triglycerides per mouse) via the tail vein, 2 hours before onset of the dark phase. After 15 minutes, mice were humanely killed by cervical dislocation and perfused with ice-cold phosphate-buffered saline through the heart. Thereafter, hearts 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- and [^{14}C]deoxyglucose-derived radioactivity was quantified and expressed per heart or per gram of heart tissue.

Tissue Collection, RNA Isolation and Real-time Quantitative Polymerase Chain Reaction

The mice were anesthetized, blood was drawn from the left ventricle and the mice were perfused with saline solution (0.9% sodium chloride in water) under physiological pressure. The LV of the heart was dissected, snap frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted using RNeasy Plus Universal Mini Kit (catalog No. 73404 from Qiagen) according to the manufacturer's instructions. Complementary DNA was synthesized with a high-capacity complementary DNA reverse transcription kit (catalog No. 4374966; Applied Biosystems). Reverse-transcription–polymerase chain reaction analysis was performed as single-plex quantitative polymerase chain reaction with TaqMan Fast Advanced Master Mix and predesigned TaqMan Gene Expression Assays (Applied Biosystems): *Cd36* (Mm01135198_m1), *Glut1* (*Slc2a1*; Mm00441480_m1), *Glut4* (*Slc2a4*; Mm00436615_m1), *b-Mhc* (*Myh7*; Mm01 319006_g1), *a-Mhc* (*Myh6*; Mm00440359_m1), *Anp* (*Nppa*; Mm01255747_g1), *Bnp* (*Nppb*; Mm00435304_g1), and *Hprt1* (Mm00446968_m1). The analyses were run in Viia 7 Real-time PCR System (Applied Biosystems). Data were normalized to the reference gene *Hprt1* and gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting

Frozen LV tissue was homogenized, followed by protein extraction using Qproteome Mammalian Protein Prep Kit (Qiagen). The lysed samples were centrifuged at 14 000*g* for 10 minutes at 4 °C. The supernatant was collected and quantified using Pierce BCA Protein Assay Kit (ThermoFisher). Equal amounts of total protein lysate were loaded onto NuPAGE 4% to 12%, Bis-Tris (Invitrogen) or Novex 4% to 20%, Tris-Glycine (Invitrogen) and underwent electrophoresis at 150 V for 70 minutes. The separated proteins were transferred to poly (vinylidene fluoride) membrane (Bio-Rad) at 100 V for 50 minutes. After blocking with TBS-T (Tris-buffer saline containing 0.1% Tween 20) containing 5% powdered milk (β-myosin heavy chain [βMHC]) or 5% bovine serum albumin (BSA) (atrial natriuretic peptide

[ANP]; brain natriuretic peptide [BNP]) for 60 minutes, the membranes were incubated with primary antibodies overnight at 4 °C and then with the corresponding horseradish peroxidase–conjugated secondary antibody at room temperature. Immunoblots were visualized with Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore) and a ChemiDoc Touch Imaging System (BioRad). Bands were quantified with Image Lab Software (Bio-Rad) and normalized to HPRT1. The commercially available antibodies used were anti-MYH7β (Ab172967, Abcam, RRID:AB_2892244), dilution 1:1000 (5% powdered milk/TBST); anti-BNP (Ab236101, Abcam, temporary RRID: AB_2923096), dilution 1:500 (3% BSA/TBST); anti-ANP (Ab262703, Abcam, temporary RRID:AB_2923097), dilution 1:1000 (3% BSA/TBST); and anti-HPRT1 (Ab109021, Abcam, RRID:AB_10866312), dilution 1:10 000 (3% BSA/ TBST).

Statistical Analysis

Statistical evaluations were performed with Prism software (GraphPad Software Inc, version 9.1.0). All variables were tested for normal distribution (Shapiro-Wilk normality test) and equality of variances (2 groups by F test and 3 groups by Brown-Forsythe test). For variables that passed normality and equal variance test, 2-group comparisons were performed by 2-tailed unpaired t test and 3-group comparison with one-way analysis of variance followed by Tukey post hoc test. Data that did not pass normality or equal variance tests were analyzed using Mann-Whitney U test (2 groups) or Kruskal-Wallis test followed by the Dunn hoc test. P values of less than .05 were considered statistically significant. Unless otherwise specified, results are represented as mean ± SEM.

Results

Castration Reduces the Relative Weight of the Heart

To address the effects of castration (orchiectomy, ORX) on heart weight, we sham-operated and castrated mice and

treated the mice with vehicle or a physiological replacement dose of testosterone (11-12 mice/group). The castration reduced and testosterone regimen normalized the weight of the androgen-sensitive seminal vesicles (Fig. 1A). Castration for 3 weeks reduced, or tended to reduce, body weight, heart weight as well as the heart weight adjusted to body weight, and these variables were increased by testosterone replacement (Fig. 1B-1D).

Castration Impairs Cardiac Performance During Stress

To investigate the effect of castration on cardiac function, male mice were castrated or sham-operated and cardiac function was evaluated 4 weeks later by echocardiography during rest and during pharmacological stress induced by intraperitoneal injection of dobutamine (Fig. 2A-2F). There were no significant differences in cardiac functional variables between the castrated (n = 8) and testes-intact (n = 10) mice under unstressed conditions. During stress, castrated mice showed a significantly reduced heart rate (−7%; $P < .01$; see Fig. 2A) and stroke volume (−18%; $P < .05$; see Fig. 2D) and, consequently, a reduced cardiac output (−24%; $P < .01$; see Fig. 2E) and cardiac index, that is, cardiac output normalized to body weight (−14%; $P < .05$; see Fig. 2F).

Castration Alters Cardiac Energy Substrate Uptake

Altered cardiac structure and function is frequently associated with changes in cardiac metabolism, such as reduced fatty acid β-oxidation and increased glucose utilization, which are mirrored by changes in substrate uptake by the heart [5]. To test the hypothesis that castration shifts substrate uptake by the heart from lipids to glucose, we determined the heart-specific uptake of fatty acids (derived from intravenously injected lipoprotein-like particles labeled with glycerol tri[³H] oleate) and glucose ([¹⁴C]deoxyglucose) in sham-operated and castrated mice treated with vehicle or a physiological replacement dose of testosterone (8 mice/group). Castration reduced uptake of fatty acids expressed per whole heart (−35%;
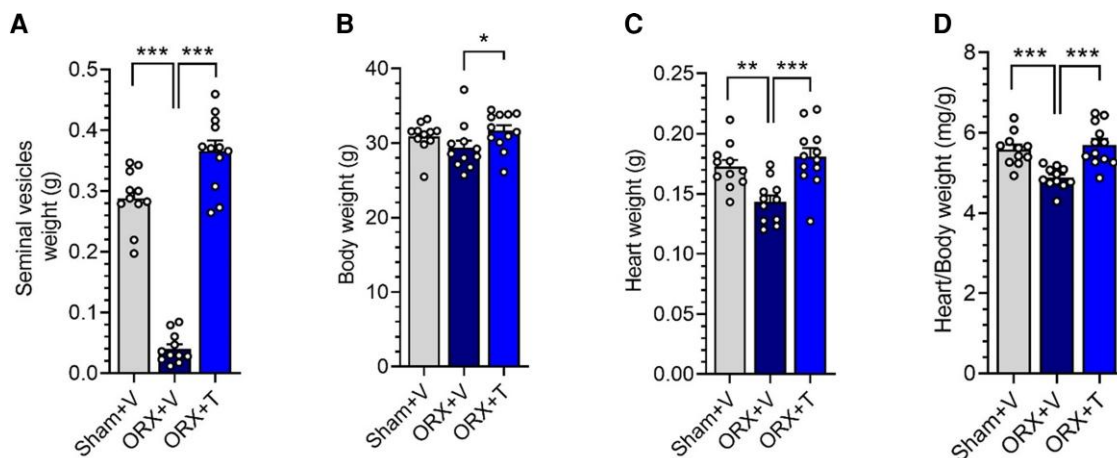

Figure 1. Castration reduces relative weight of the heart. At age 14 weeks, male mice were sham-operated (Sham) or castrated (ORX) and treated with vehicle (V) or a physiological dose of testosterone (T). Tissues were collected and weighed 3 weeks later. A, Weight of seminal vesicles; B, body weight; C, heart weight and D, heart weight normalized to body weight. Bars indicate means, error bars indicate SEM, and circles represent individual mice. n = 11 (Sham + V, ORX + V) or 12 (ORX + T) mice per group. Data were analyzed by one-way analysis of variance followed by Tukey post hoc test (A, C, and D) or Kruskal-Wallis test followed by Dunn hoc test (B). *$P < .05$, **$P < .01$, ***$P < .001$.
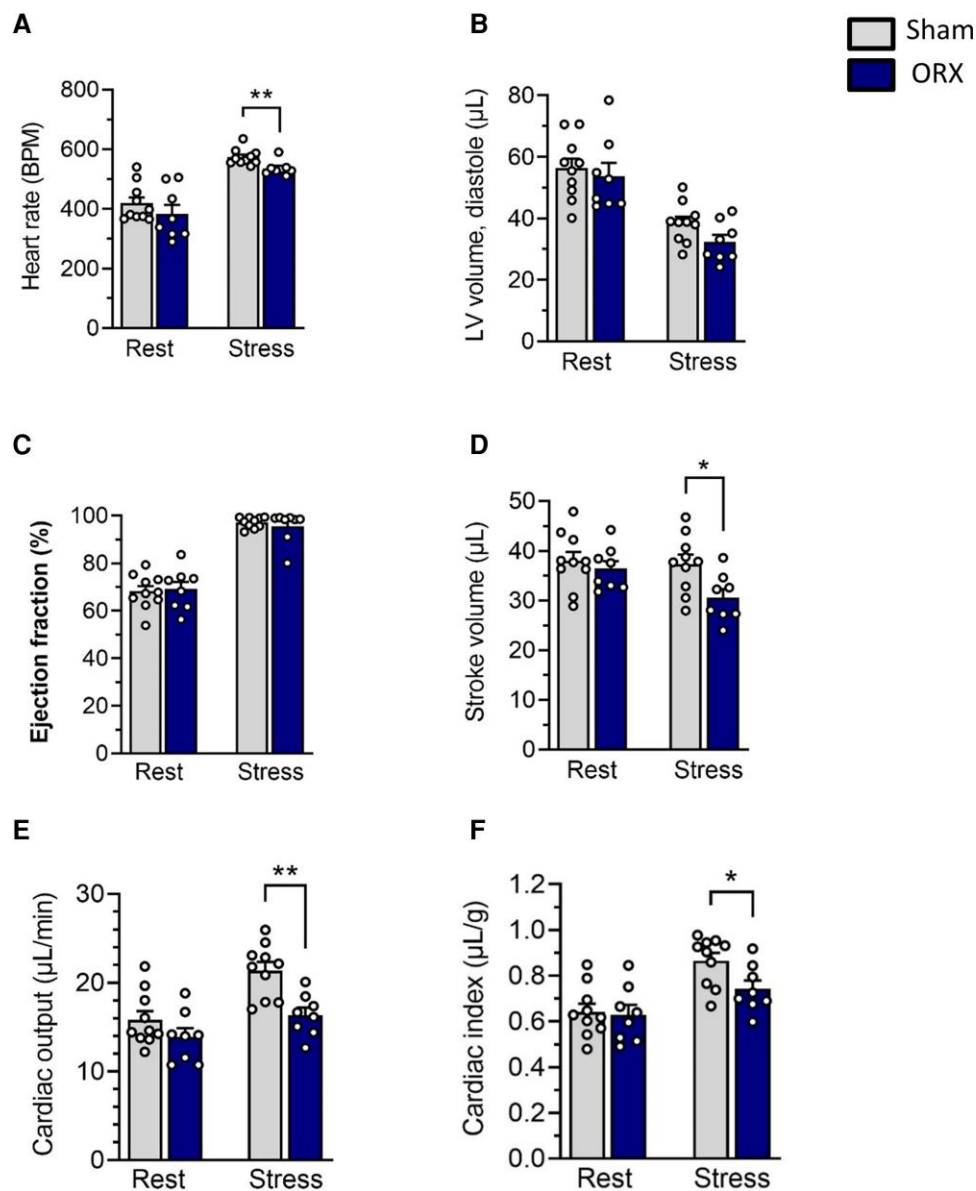
Figure 2. Castration impairs cardiac performance during stress. Male mice were sham-operated (Sham; n = 10) or castrated (ORX; n = 8) at age 9 weeks. Cardiac function was evaluated with echocardiography during rest and pharmacological stress (a single intraperitoneal injection of dobutamin) 4 weeks after surgery. Bars indicate means, error bars indicate SEM, and circles represent individual mice. BPM, beats per minute; LV, left ventricular. Data were analyzed by Mann-Whitney U test; ORX were compared with Sham in Rest and Stress, respectively. *$P < .05$, **$P < .01$.

$P < .05$; Fig. 3A) with a similar tendency per gram heart (-26%; $P = .10$; Fig. 3B), and testosterone replacement had the opposite effects. Further, castrated mice showed increased uptake of glucose in the whole heart ($+35\%$; $P < .05$; Fig. 3C) and per gram heart ($+56\%$; $P < .05$; Fig. 3D), which was similarly normalized by testosterone replacement. Thus, our data indicate that castration induces a relative switch of metabolic substrate uptake in the heart from lipids toward glucose whereas testosterone replacement prevents this effect.

Energy substrate uptake into cardiomyocytes occurs by facilitated diffusion through specific membrane-associated proteins: CD36 for fatty acids, and GLUT1 and GLUT4 for glucose (5). By analyzing messenger RNA (mRNA) levels of these transporters in the LV, we found that castration

decreased *Cd36* mRNA, while there were no statistically significant effects on *Glut1 or Glut4* mRNA levels (Fig. 4A).

Castration Induces Expression of Fetal Genes in the Heart

In response to stress, the postnatal heart returns to the fetal gene program to support cardiac structure and function [6]. The β isoform of myosin heavy chain (βMHC) is the predominant isoform in the developing mouse ventricle, but during early postnatal development it is replaced by αMHC. To test whether the MHC isoform changes in castrated mice, we measured the expression of MHC genes in the heart. Indeed, castrated mice showed a large increase in mRNA expression of *b-Mhc* ($+9$-fold; $P < .001$) and a small reduction in *a-Mhc*

mRNA (-8%; $P < .05$) compared to sham-operated mice (18-20 mice/group). Further, the hearts of castrated mice showed increased mRNA levels of both ANP (*Anp*; $+54\%$; $P < .01$) and BNP (*Bnp*; $+24\%$; $P < .05$), which are additional features of the fetal gene program (Fig. 4B). We next addressed whether castration also regulated cardiac βMHC, ANP, and BNP at the protein level (7-12 mice/group). Indeed, we found that βMHC ($+216\%$ in castrated mice; $P < .05$), ANP ($+131\%$; $P < .05$), and BNP ($+108\%$; $P < .05$) protein levels all were increased by castration and

to the same extent reduced by testosterone replacement (Fig. 5).

Discussion

In the present study, we report that castration of male mice results in a relative switch of metabolic substrate uptake in the heart from lipids toward glucose whereas testosterone replacement had the opposite effect. This switch in substrate uptake was associated with expression of the fetal gene program, including a strong increase in the expression of βMHC, the fetal isoform of MHC. Further, castration reduced relative heart weight and impaired cardiac performance during pharmacological stress.

To our knowledge, this is the first study showing that castration alters metabolic substrate uptake in the heart. In line with a negative effect of androgens on cardiac glucose uptake, female mice (with much lower androgen levels) have a higher glucose uptake in the heart compared to male mice [16]. The use of energy substrates in the heart is determined at multiple
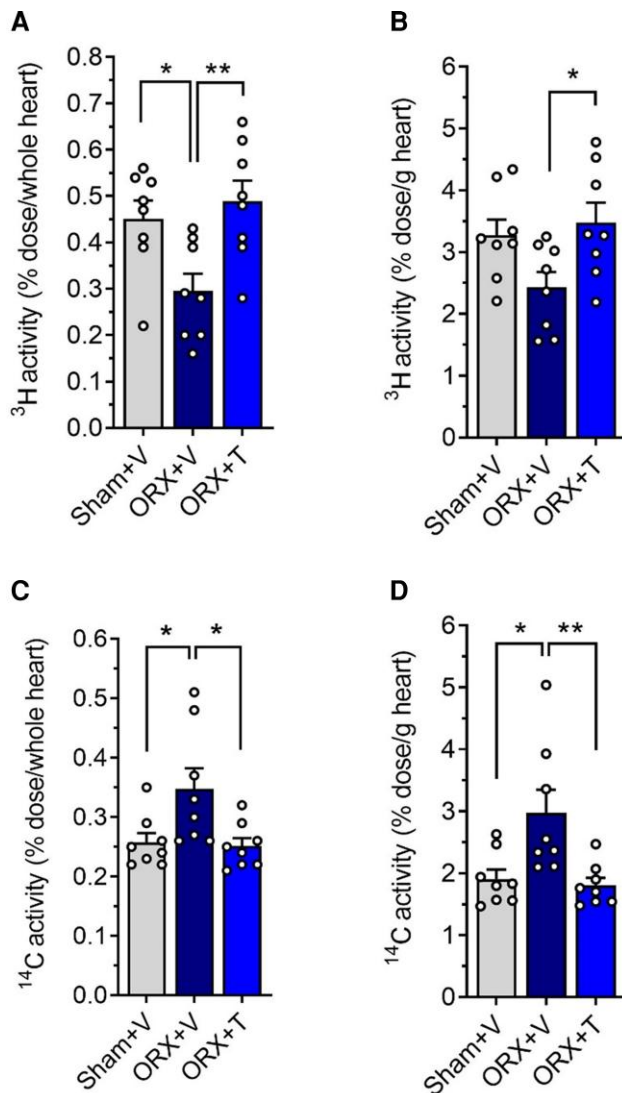
Figure 3. Castration alters cardiac energy substrate uptake. At age 10 to 11 weeks, male mice were sham-operated (Sham) or castrated (ORX) and treated with vehicle (V) or a physiological dose of testosterone (T); n = 8 mice per group. Three weeks after surgery, mice were fasted for 6 hours and injected intravenously with glycerol tri[^{3}H]oleate-labeled triglyceride-rich lipoprotein-like emulsion particles and [^{14}C] deoxyglucose. After 15 minutes, mice were euthanized and uptake of radioactivity in the heart was determined. A and B, Uptake of glycerol tri[^{3}H]oleate-derived radioactivity A, per heart, and B, per gram of heart tissue. C and D, Uptake of [^{14}C]deoxyglucose-derived radioactivity C, per heart, and D, per gram of heart tissue. Bars indicate means, error bars indicate SEM, and circles represent individual mice. Data were analyzed by one-way analysis of variance followed by Tukey post hoc test. *$P < .05$, **$P < .01$.
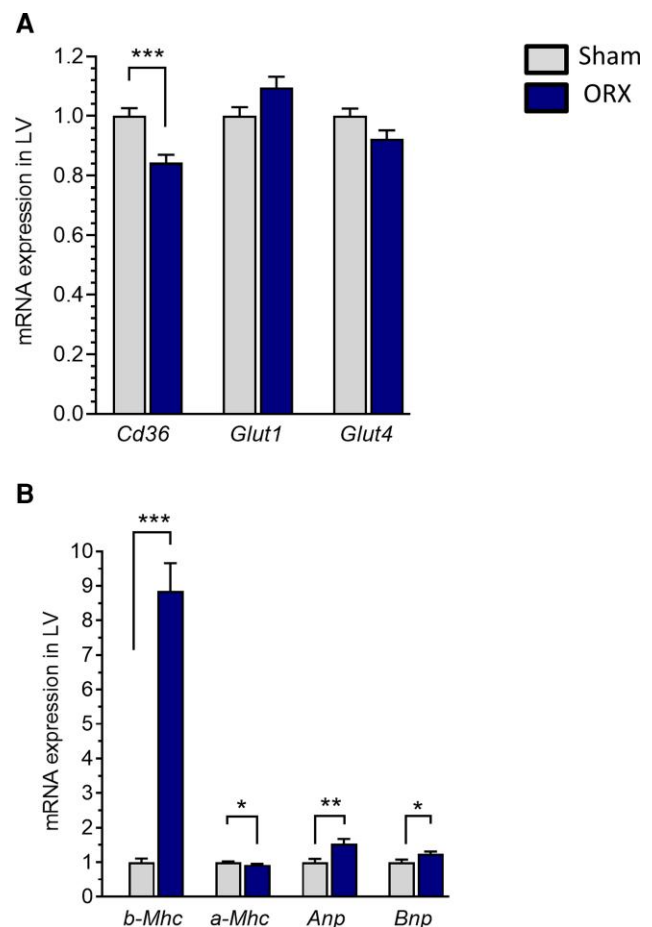
Figure 4. Castration induces messenger RNA (mRNA) expression of fetal genes in the heart. Male mice were sham-operated (Sham; n = 18-20) or castrated (ORX; n = 18-20) at age 11 to 13 weeks and the left ventricle was collected 3 weeks later. A, mRNA levels of genes involved in lipid (*Cd36*) and glucose transport (*Glut1* and *Glut4*) and B, expressed in the fetal gene program (*b-Mhc*, *Anp*, and *Bnp*) as well as *a-Mhc* (that replaces *b-Mhc* during postnatal development) were analyzed by quantitative polymerase chain reaction. Expression levels were normalized against the reference gene *Hprt1*. Bars indicate means, error bars indicate SEM. LV, left ventricle. Data were analyzed by *t* test or Mann-Whitney *U* test. *$P < .05$, **$P < .01$, ***$P < .001$ vs Sham.

levels, but the intracellular availability of substrates is an important parameter governing their use for energy provision [5]. In accordance with the observed reduction in relative fatty acid uptake, we found a small decrease in *Cd36* mRNA in the LV of castrated males. An increase in glucose metabolism at the onset of heart disease is part of an adaptive mechanism to protect the heart from injury [17]. However, this tilted preference of cardiac substrates (ie, fatty acids vs glucose) is associated with impaired cardiac contractile function, regardless of the underlying cause [5] and may lead to decompensation and heart failure progression [17]. Thus, it is possible that metabolic remodeling contributes to reduced cardiac performance in testosterone deficiency, which should be addressed in future studies.

βMHC, which is the predominant MHC isoform in the developing mouse ventricle, is replaced by αMHC during early postnatal development [6]. In the present study, we found a strong upregulation of *b-Mhc* and a modest reduction of *a-Mhc* mRNA in castrated mice. This finding is in accordance with previous reports of increased βMHC [18] and reduced α/βMHC protein ratio in the hearts of castrated rats [19]. Further, expression of both ANP and BNP, also considered to be a feature of the fetal gene program as well as markers of cardiac stress, was higher in the LVs of castrated mice.

Previous studies showed a direct hypertrophic effect of testosterone on the myocardium (1). In accordance, we found that castration reduces the relative heart weight. Further, we found here that short-term castration of male mice significantly impairs cardiac performance during pharmacological stress, but not at rest. In line with our data, most other studies found no effect of castration on in vivo systolic variables in unstressed male mice and rats [8, 9]. However, LV filling and function at controlled pressure levels are impaired in hearts of prepubertally gonadectomized male rats, and cardiomyocytes isolated from castrated rats show reduced cardiomyocyte shortening as well as increased relaxation [9, 18, 20]. Indeed, both clinical and preclinical data suggest that testosterone influences cardiac contractility [20], but it is

possible that effects on resting cardiac function requires more long-term and/or prepubertal castration as compared to the short-term castration of 9-week-old mice performed in our study. Besides the young age at castration, our echocardiographic study is also limited by a small sample size. Contrasting the unstressed condition, castrated mice showed relatively reduced stroke volume, heart rate, and cardiac output during dobutamine stress. Dobutamine is a synthetic catecholamine that acts on α1, β1, and β2 adrenergic receptors and stimulation of these receptors in the heart produces a relatively strong, additive inotropic effect and a relatively weak chronotropic effect. In the vasculature, α1-mediated vasoconstriction balances the β2-mediated vasodilatation [21]. Similar to other studies [13, 14], we applied a heart rate criterion for dobutamine response; we cannot exclude that this may severely affect our conclusions, although the same number of mice were excluded from the Sham and ORX groups (4/group). In line with reduced cardiac output of castrated mice during stress in the present study, testosterone has been shown to augment cardiac contractile responses to α1- and β1 adrenoceptor stimulation [22].

Further studies will be needed to understand the mechanism(s) linking testosterone deficiency to metabolic remodeling of the heart. It has been suggested that metabolic signals precede and trigger the return to the fetal gene program, and long-term increases in glucose supply and utilization are associated with a shift to a fetal transcriptional program in adult cardiomyocytes [6]. Glucose availability correlates with MHC isoform switching under all conditions investigated, likely as a mechanism to reduce energy consumption while burning less energy-providing glucose [6]. The amount of *b-Mhc* mRNA increases in hearts subjected to both increased or decreased mechanical stress, and the remodeling process of the atrophied heart also includes a return to the fetal gene program [23]. In the setting of castration, both direct hormonal effects and changed cardiac load may potentially underlie the metabolic/gene expression switch. A role for indirect metabolic effects, for example, via changes of glucose/lipid
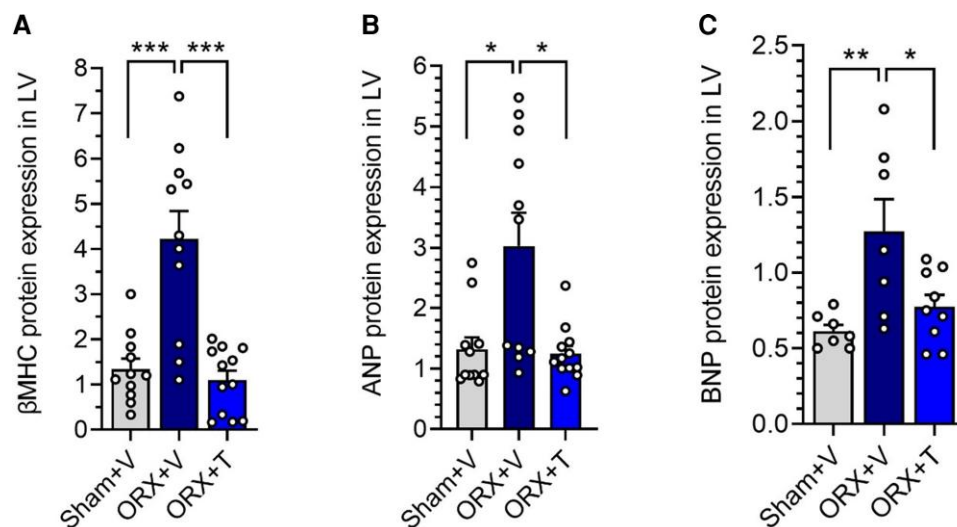

Figure 5. Castration induces protein expression of fetal genes in the heart. At age 14 weeks, male mice were sham-operated (Sham) or castrated (ORX) and treated with vehicle (V) or a physiological dose of testosterone (T). Tissues were collected 3 weeks later and A, β-Myosin heavy chain (βMHC); B, atrial natriuretic peptide (ANP); and C, brain natriuretic peptide (BNP) protein levels quantified. Protein levels were normalized against the reference protein HPRT1. Bars indicate means, error bars indicate SEM, circles represent individual mice and LV, left ventricle. n = 7 to 12 mice per group. Data were analyzed by one-way analysis of variance followed by Tukey post hoc test. *P < .05, **P < .01, ***P < .001.

metabolism by testosterone [24], is also plausible as factors such as adipose tissue depot size, lipolysis, and plasma lipid delivery to the heart are important determinants of myocardial substrate metabolism and function [6]. Whether the effects of testosterone are mediated via the androgen receptor, conversion to estradiol, gonadotropin signaling, or other pathways remains unclear and requires further study.

There are now several studies linking androgen deprivation therapy in prostate cancer patients to increased risk of heart failure, even in patients without any preexisting cardiovascular disease [3, 4, 25]. Further, positive effects of testosterone treatment to heart failure patients have been reported [26, 27]. An important question for future research is whether our findings on cardiac metabolism in testosterone deficiency in mice extends to humans. As interventions aimed at manipulating cellular substrate uptake to rebalance fuel supply are promising strategies to treat heart failure [5], such results may be of great interest also from a therapeutic perspective, in the setting of heart disease in testosterone deficiency.

In conclusion, castration of male mice induces metabolic remodeling and expression of the fetal gene program in the heart, in association with a reduced cardiac response to pharmacological stress. These findings may be relevant for the selection of treatment strategies for heart failure in the setting of testosterone deficiency.

Disclosures

The authors have nothing to disclose.

Data Availability

Some or all data sets generated during and/or analyzed during the present study are not publicly available but are available from the corresponding author on reasonable request.

Current Affiliation

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