Anti-Androgenic Therapy with Finasteride Attenuates Cardiac Hypertrophy and Left Ventricular Dysfunction

Running title: Zwadlo et al.; Finasteride Attenuates Cardiac Hypertrophy

Carolin Zwadlo, MD1*; Elisa Schmidtmann, BS1*; Malgorzata Szaroszyk, MS1*; Badder Kattih, MD1; Natali Froese, PhD1; Hebke Hinz, BS1; Jan Dieter Schmitto, MD2; Julian Widder, MD1, Sandor Batkai, MD, PhD3; Heike Bähre, PhD5; Volkhard Kaever, PhD5; Thomas Thum, MD, PhD3,4; Johann Bauersachs, MD1; Joerg Heineke, MD1

1Medizinische Hochschule Hannover, Klinik für Kardiologie und Angiologie, Hanover, Germany; 2Klinik für Herz-, Thorax-, Transplantations- und Gefäßchirurgie, Hannover, Germany; 3Institut für Molekulare und Translationale Therapiestrategien (IMTTS), Hannover, Germany; 4National Heart and Lung Institute, Imperial College, London, United Kingdom; 5Medizinische Hochschule Hannover, Zentrale Forschungseinrichtung Metabolomics, Institut für Pharmakologie, Hannover, Germany

*contributed equally

Address for Correspondence:
Joerg Heineke, MD
Medizinische Hochschule Hannover, Klinik für Kardiologie und Angiologie
Experimentelle Kardiologie, Rebirth – Cluster of Excellence
Carl-Neuberg-Str.1, 30625 Hannover, Germany
Tel: ++49-511-532-3079
Fax: ++49-511-532-5412
E-mail: Heineke.Joerg@Mh-Hannover.de

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Abstract

Background—Compared to men, women have a better prognosis when suffering from aortic valve stenosis, hypertrophic cardiomyopathy or heart failure. Recent data suggest that androgens like testosterone or the more potent dihydrotestosterone (DHT) contribute to the development of cardiac hypertrophy and failure. Therefore, we analyzed whether anti-androgenic therapy with finasteride, which inhibits the generation of DHT by the enzyme 5α-reductase, improves pathological ventricular remodeling and heart failure.

Methods and Results—We found a strongly induced expression of all three isoforms of the 5α-reductase (Srd5α1-3) in human and mouse hearts with pathological hypertrophy, which was associated with increased myocardial accumulation of DHT. Starting one week after the induction of pressure overload by transaortic constriction, mice were treated with finasteride for two weeks. Cardiac function, hypertrophy, dilation and fibrosis were markedly improved in response to finasteride treatment in male, but also in female mice. In addition, finasteride also very effectively improved cardiac function and mortality after long-term pressure overload and prevented disease progression in cardiomyopathic mice with myocardial Gαq overexpression. Mechanistically, finasteride by decreasing DHT potently inhibited hypertrophy and Akt dependent pro-hypertrophic signaling in isolated cardiac myocytes, while introduction of constitutively active Akt blunted these effects of finasteride.

Conclusions—Finasteride, which is currently used in patients to treat prostate disease, potently reverses pathological cardiac hypertrophy and dysfunction in mice, and might be a therapeutic option for heart failure.

Key words: hypertrophy, heart failure, hormones
Introduction

Around five million Americans suffer from chronic heart failure (CHF)\textsuperscript{1}. The most common causes for the development of CHF are chronic arterial hypertension, myocardial hypertrophy and previous myocardial infarction\textsuperscript{1}. The progression of heart failure is driven by pathological ventricular remodeling processes, which mainly involve cardiomyocyte hypertrophy, myocardial fibrosis and profound changes in gene-expression that together lead to left ventricular dilation and systolic as well as diastolic dysfunction over time\textsuperscript{2-4}. Despite recent therapeutic advances, the mortality of CHF remains high, and - a fact that is somewhat neglected - strikingly different between women and men. Women with heart failure have a lower mortality compared to men and also a better prognosis when suffering from hypertension, aortic valve stenosis or hypertrophic cardiomyopathy\textsuperscript{1,5}. Sex hormones might account for these differences and indeed the expression of androgen as well as estrogen receptors was found in male and female hearts, implying that estrogen and androgens could directly act on the myocardium\textsuperscript{6,7}. While in general estrogen has been deemed cardioprotective, large randomized and controlled studies have failed to demonstrate beneficial effects of estrogen therapy in postmenopausal women\textsuperscript{8,9}. As a consequence, it is now proposed that the rise in cardiovascular mortality in women after the onset of menopause might be caused by an increased ovarian production of testosterone\textsuperscript{10}.

Testosterone and its highly active metabolite dihydrotestosterone (DHT) operate by binding to the androgen receptor, which can act as a ligand dependent transcription factor, but also in a so called “non-genomic” fashion by potently influencing cellular signal-transduction\textsuperscript{11}. Recent data point towards a pro-hypertrophic effect of testosterone and DHT on isolated cardiomyocytes and in mice\textsuperscript{7,12,13}. However, it remains unclear whether inhibition of androgens might be beneficial during heart failure or hypertrophy, and how anti-androgenic therapy should
be conducted, since complete blockade of androgen action is typically associated with severe side-effects\textsuperscript{14}. In this study, we analyzed whether treatment with finasteride, a drug that is currently used for the therapy of benign prostate hyperplasia reduces cardiac hypertrophy and failure in mice\textsuperscript{14}. Finasteride specifically inhibits isoforms 2 and 3 of the 5-\alpha-reductase, the enzyme that converts testosterone to DHT, which is the most potent androgen of the body and about 10-fold more potent than testosterone\textsuperscript{14-16}. We show here that finasteride by reducing DHT levels, very effectively reverses pathological hypertrophy, fibrosis and left ventricular dysfunction in mice of both sexes.

Methods

An expanded Methods section is provided in the Supplemental Material. Transverse aortic constriction (TAC) was performed in 8- to 10 week old C57/Bl6N mice (Charles River) around a 25-gauge needle as previously described\textsuperscript{17}. Hemizygous transgenic mice expressing the entire coding region of the \textit{Gaq} protein under the control of the \alpha-myosin heavy chain promoter have been described before and were obtained from the Jackson laboratory\textsuperscript{18}. Littermate mice not carrying the transgene (wild-type, WT) were used as control. Echocardiography was performed with a linear 30MHz transducer (Visualsonics, Toronto, Canada) in mice that were sedated with 1-1.5\% isoflurane. LV end-diastolic area (LVEDA) and end-systolic area (LVESA) were recorded. Fractional area change was calculated as [(LVEDA-LVESAF/LVEDA) x 100. Left ventricular pressures were assessed with a microtip pressure-volume catheter (PVR-1000, Millar Instruments, Houston, TX) inserted through the cardiac apex during anesthesia with 2\% isoflurane. Finasteride (Sigma Aldrich, St. Louis, MO) was administered daily by oral gavage as suspension in 0.5\% methyl cellulose at a dose of 25mg/kg/day. At the end of the experiments,
total body and lung and heart weights were determined. All animal procedures were approved by our local state authorities.

Neonatal rat ventricular cardiomyocytes (NRCMs) were isolated from 1-3 day old Sprague-Dawley rats by Percoll density gradient centrifugation as previously described19. On the day after isolation, the cells were switched to serum free media and incubated with phenylephrine (20μM), fetal bovine serum (FBS, 2%), testosterone (1μM), dihydrotestosterone (DHT, 1μM) or vehicle with or without the addition of finasteride (25μM) and/or flutamide (10μM) as indicated. If needed, adenoviral vectors encoding for either β-galactosidase (Ad.Control), constitutive active proteinkinase B/Akt (Ad.caAkt) or dominant negative Akt (Ad.dnAkt) were added to the cells for two hours on the day after isolation, and stimulation with FBS and finasteride was subsequently conducted. Cell size was determined by planimetry after immuno-fluorescence staining for Troponin I (H-170, Santa Cruz Biotechnology).

RNA from cultured cardiomyocytes, mouse hearts or human hearts was isolated with the Trifast reagent (Peqlab, Erlangen, Germany). The human heart samples used in this study were in part described previously and obtained after death, explantation or left ventricular assist device implantation with previous institutional review board approval of the Massachusetts General Hospital and the Medizinische Hochschule Hannover20. cDNA synthesis and quantitative PCR was performed using standard procedures and the MX4000 multiplex QPCR system from Stratagene. The PCR primers are listed in the Supplemental Table.

Western blotting was performed using standard procedures, as described17.

Transversal frozen sections of the myocardium were generated to measure cardiomyocyte cross-sectional area after staining the cardiomyocyte cell membrane with tetramethyl rhodamine
isothiocyanate-conjugated wheat-germ agglutinin (WGA, Sigma Aldrich). Fibrosis was quantified with the Sirius Red staining method.

We used the AbsoluteIDQ Stero17 kit (Biocrates, Innsbruck, Austria) to assess the concentrations of testosterone, DHT, estradiol and androstendione in mouse serum. The procedure uses a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method and was conducted according to the manufacturer’s instructions as described21.

Statistical Analysis

All values are presented as means ± SEM. The Mann-Whitney test was used for comparisons between two groups. Differences between three or more groups were analyzed with the Kruskal-Wallis test followed by Dunn’s multiple comparison’s test. Differences between three or more groups in cardiomyocyte cell size in vitro were analyzed by one-way analysis of variance followed by Sidak’s multiple comparisons test. Mouse mortality after TAC surgery was analyzed by the log-rank test. A two-tailed P value of less than 0.05 was considered significant. All statistics were calculated with the Graph Pad Prism 6 software.

Results

The expression of 5-α-reductase isoforms is induced in cardiac hypertrophy

Finasteride inhibits isoforms 2 and 3 of the 5-alpha-reductase enzyme that converts testosterone to the more active metabolite DHT. We assessed the expression of all three isoforms of this enzyme (Srd5a1-Srd5a3) in different pathological conditions associated with cardiomyocyte hypertrophy. Absolute quantification in human myocardial samples revealed Srd5a3 as the most abundantly expressed isoform of the heart (Figure1A). Srd5a3 mRNA was significantly induced in human hearts with hypertrophy, while it was unchanged in hearts from patients with endstage
heart failure. The expression of Srd5a1 and 2 was significantly increased in both conditions. In mouse hearts, the expression of all three Srd5a isoforms was induced two and six weeks after the induction of pressure overload by TAC surgery (Figure 1B). Western blot analysis revealed enhanced protein abundance of Srd5a3 in mouse hearts after 2, 6 or 12 weeks of pressure overload (Figure 1C, D). In conclusion, while finasteride sensitive Srd5a3 is predominant in the heart, all three Srd5a isoforms are induced in cardiac hypertrophy.

**Finasteride reduces increased myocardial DHT in the hypertrophic myocardium**

Because isoforms of the DHT producing enzyme 5-α-reductase were upregulated in cardiac hypertrophy, we measured the expression of Mt1, Odc1 and Axin1 mRNA as DHT responsive genes by real-time PCR in the myocardium of mice after TAC or sham surgery subjected to daily treatment with finasteride (starting one week after surgery for two weeks)22. Male mice exerted mildly induced expression of Mt1, Odc1 and Axin1 after TAC (Figure 2A). In response to finasteride, Mt1 and Odc1 mRNA were strongly downregulated - even below the level observed in sham operated mice - after TAC, while Axin1 expression was not influenced. We also found a robust myocardial induction of Mt1, Odc1 and Axin1 after TAC in female mice, which was attenuated by finasteride treatment (Figure 2B). To measure myocardial DHT abundance more directly in myocardial samples, we used an antibody raised against protein-bound DHT. In Western blots of isolated NRCMs probed with this antibody, we identified a specific protein band at 25kDa that was markedly diminished in cardiomyocytes incubated with finasteride, indicating that this band represented a DHT-protein complex (Figure 2C). We found an induction of this DHT-protein complex after TAC in untreated male and female, but not in finasteride treated mice (Figure 2D). Interestingly, we also observed increased DHT-protein in...
human hypertrophic hearts (Figure 2E). In summary, we found strong evidence for increased myocardial DHT levels in hypertrophic hearts that were effectively reduced by finasteride.

**Finasteride inhibits cardiomyocyte hypertrophy in vitro**

We first investigated the functional impact of finasteride on hypertrophy in NRCMs. As depicted in Figure 3A and B, phenylephrine and FBS led to significant cardiomyocyte hypertrophy with increased cell size and induction of BNP mRNA expression. The increase in cell size in response to both stimuli was significantly blunted by finasteride as was the increase in BNP in response to FBS, but not in response to phenylephrine. Because we hypothesized that finasteride reduced cardiomyocyte hypertrophy through a reduction of DHT abundance, we tested whether DHT induced cardiomyocyte hypertrophy and whether finasteride still inhibited hypertrophy when DHT was present in excess. As shown in Figure 3C and D, testosterone and DHT both induced cardiomyocyte hypertrophy, while only the testosterone induced cell growth, but not that by DHT could be inhibited by finasteride. Incubation of NRCM with the androgen receptor inhibitor flutamide blocked phenylephrine induced hypertrophy to a similar extent as finasteride (Figure 3E), which also did not exert an additional anti-hypertrophic effect in the presence of flutamide, suggesting that both agents act through a similar cellular target, i.e. androgen induced signaling. Thus, finasteride efficiently inhibits cardiomyocyte hypertrophy induced by different stimuli, depending on its capability to reduce DHT levels and androgen receptor activation.

**Finasteride therapy inhibits cardiac hypertrophy and dysfunction in male and female mice**

To analyze whether finasteride therapy inhibits pathological cardiac hypertrophy and cardiac dysfunction during pressure overload in mice *in vivo*, we started daily treatment with finasteride one week after TAC or sham surgery and continued it for two weeks. In male mice, finasteride administration inhibited cardiac hypertrophy after TAC compared to untreated mice as evident
from a markedly reduced heart weight/body weight ratio (HW/BW, Supplemental Figure 1, Figure 4A), a reduced cardiomyocyte cross-sectional area (Figure 4D) and blunted upregulation of ANP and BNP mRNA expression (Figure 4C). Induction of RCAN1.4 expression, a marker of calcineurin/NFAT activation, was also reduced in finasteride treated mice (Figure 4C). Echocardiographic examination revealed an improved left ventricular systolic function (measured as fractional area change, Figure 4B) and a significantly reduced cardiac dilation (enddiastolic area after TAC; 13.7±0.7 versus 11±0.6 mm²) in response to finasteride treatment. Fibrosis was reduced in TAC mice after finasteride administration, which was supported by a reduced collagen3 mRNA expression (Figure 4C and E, F). Finasteride had no effect in sham operated mice (Figure 4A-E). As expected and shown in Figure 4G, finasteride dramatically reduced DHT serum levels, while it had no significant impact on testosterone or androstenedione (Supplemental Figure 2B). Androgen receptor expression was not significantly different among groups (Supplemental Figure 2A).

Although female mice in general have far lower systemic levels of testosterone and DHT (both hormones were under the level of detection of our assay in serum, data not shown), we still obtained evidence of local DHT accumulation in the myocardium of female mice that were reduced by finasteride (see above, Figure 2B, D). Therefore, we also analyzed whether cardiac remodeling can be positively affected by the same finasteride treatment regimen in female as in male mice. This was indeed the case: HW/BW, cardiomyocyte cross-sectional area, cardiac systolic function as well as myocardial fibrosis and cardiac dilation were significantly improved upon treatment with finasteride (Figure 5A-F, and data not shown). In addition, the induction of embryonic genes (ANP, BNP) as well as collagen3 and RCAN1.4 was blunted in finasteride treated female mice after TAC (Supplemental Figure 3A). We determined serum estradiol
levels, in order to rule out that finasteride treatment led to an increased abundance of estradiol, but no difference was found among groups (Supplemental Figure 3B).

**Finasteride improves cardiac function and reduces mortality in heart failure**

To assess whether finasteride treatment is also beneficial in advanced myocardial disease, we started it 21 days after TAC or sham surgery in male mice and continued for 32 days (Figure 6A). We found a significantly reduced mortality after TAC in the finasteride treated mice (Figure 6B). After four weeks of finasteride treatment, mice had a better systolic function compared to untreated mice (Figure 6C). Millar-catheterization revealed an improved cardiac relaxation (dp/dt min and Tau) and a trend towards better contractility (dp/dt max) after TAC (Figure 6D). There was no difference in these parameters in sham treated mice. Intraventricular systolic blood pressure rose to a similar extent after TAC in treated and untreated mice, indicating that both groups of mice were exposed to a similar amount of pressure overload (Supplemental Figure 2C). In addition, systolic blood pressure was similar in untreated and treated sham operated mice, thus excluding a direct effect of finasteride on systolic blood pressure (Supplemental Figure 2C). Because androgens (especially testosterone) positively regulate skeletal muscle mass, we analyzed skeletal muscle weights in the different treatment groups, but no differences were observed (Supplemental Figure 4A).

**Finasteride improves cardiac function in Gαq triggered cardiomyopathy**

Next, we wanted to test whether finasteride also ameliorates cardiac dysfunction in another model of heart failure. Gαq transgenic mice were described as model for cardiac failure with pronounced ventricular dilation. We started to treat male Gαq transgenic (TG) mice with finasteride at the age of eight weeks and continued the daily treatment for six weeks (Figure 7A). Untreated TG mice as well as treated and untreated WT mice were used as controls. As
demonstrated in Figure 7B, systolic function of Gαq TG mice was markedly improved by finasteride treatment. When compared to echocardiography before the treatment period, transgenic mice without treatment exerted a significant deterioration of systolic left ventricular function as well as a strong increase in cardiac dilation over time. In contrast, this was not observed in TG mice treated with finasteride (Figure 7C, D). Despite significantly improved cardiac function and the absence of ventricular dilation over time in transgenic mice treated with finasteride, only a trend towards decreased enddiastolic diameter was observed in these mice compared to untreated mice at the end of the experiment (data not shown), a fact that is likely due to the high variability in ventricular diameter in the TG mice in both groups and limited sample size. No changes were observed upon finasteride treatment in WT and TG mice regarding skeletal muscle mass and regarding blood pressure in TG mice (Supplemental Figure 2D, 4B).

**Finasteride interferes with signaling to exert its anti-hypertrophic effects**

Finally, we investigated how the reductions of DHT levels by finasteride lead to its pronounced anti-hypertrophic effects. Testosterone is known to activate signaling molecules in cardiac myocytes. Pro-hypertrophic Akt, ERK as well as downstream targets of the protein kinase mTOR (p70S6 kinase and 4-EBP1), but not mTOR itself, displayed an increased level of phosphorylation, indicating enhanced activation after TAC (Figure 8A, B and Supplemental Figure 5A). The activation of Akt and p70S6 kinase (p70S6K) was reduced in the myocardium of TAC mice after finasteride treatment. To more directly assess the link between finasteride and the inhibition of pro-hypertrophic signaling molecules, we pretreated NRCMs for 24 hours with and without finasteride and/or DHT, before we stimulated the cells with 2% FBS for 10 minutes (Figure 8C, D and Supplemental Figure 5B). While Akt and ERK were not influenced by

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finasteride at this time point, the phosphorylation of p70S6K as mTOR target was induced by FBS treatment, but not in the cells that were pretreated with finasteride. However, this effect was rescued, at least partially, when DHT was added in abundance to the culture medium, indicating that the reduction of DHT was required for finasteride triggered mTOR inhibition. In order to investigate whether modulation of signaling is responsible for the anti-hypertrophic effects of finasteride, we transduced NRCMs with an adenovirus encoding for an activated form of Akt and assessed cardiomyocyte size in response to FBS or finasteride treatment (Figure 8E and Supplemental Figure 5C). FBS induced hypertrophy was not blocked by finasteride in cardiomyocytes overexpressing activated Akt, while it could be inhibited in cells infected with control adenovirus. Akt as well as mTOR activation (measured as p70S6K phosphorylation) during FBS treatment was inhibited in the presence of control adenovirus, but finasteride did not inhibit Akt or mTOR when constitutively active Akt was present (Figure 8F, G). Even in the absence of FBS, finasteride did not inhibit cardiomyocyte hypertrophy induced by constitutively active Akt (Supplemental Figure 5E). In order to test whether Akt was required for the finasteride effects, we employed an adenovirus expressing a dominant negative (dn) Akt variant (Supplemental Figure 5F). Interestingly, finasteride did not significantly reduce FBS triggered hypertrophy in dnAkt treated cardiomyocytes (Figure 8H). Thus, finasteride blocks hypertrophy, at least in part, by targeting Akt or signaling upstream of Akt.

Discussion

We found in this study that pathological cardiac hypertrophy in mice and humans is associated with increased DHT levels in the myocardium. Finasteride potently reduced myocardial DHT and at the same time inhibited pathological cardiac remodeling and dysfunction in male and
female mice. Mechanistically, finasteride interfered with DHT dependent activation of pro-hypertrophic signaling cascades.

DHT, the most potent endogenous androgen, has a two- to five-fold higher binding capacity to the androgen receptor and a 10-fold higher potency to induce androgen receptor mediated signaling than testosterone\textsuperscript{14}. As a consequence, the main physiological function of DHT seems to be the amplification of testosterone mediated signals, for example during the development of male and female reproductive organs\textsuperscript{23}. Testosterone is converted to DHT by isoforms 1 -3 (Srd5a1-Srd5a3) of the 5-\alpha-reductase, while Srd5a3 is also involved in N-linked protein glycosylation\textsuperscript{14, 24, 25}. Each of the three enzymes is expressed at similar levels in the heart of male and female mice, and finasteride operates mainly as an inhibitor of Srd5a2 and Srd5a3\textsuperscript{26}. We demonstrated here that Srd5a3 is the predominant cardiac isoform and that the expression of all 5-\alpha-reductase isoforms is increased in human and mouse hypertrophic heart disease. We reasoned that, not unlike in the prostate where Srd5a1-3 are highly expressed and as a result DHT is the most prevalent androgen\textsuperscript{14}, increased Srd5a isoform expression in response to hypertrophic stimulation in the heart leads to increased abundance of DHT, which can be reduced by finasteride. Indeed, measurement of DHT responsive genes as well as DHT-protein complex verified this hypothesis. Although our methods to determine DHT levels in the myocardium are indirect, the detection of reduced DHT in response to finasteride argues in favor of their specificity. In support of our findings, increased levels of DHT production were previously found in \textit{ex vivo} preparation of microsomes from human hypertrophic hearts\textsuperscript{27}.

In order to mimic a clinical situation, in which no prophylactic therapy is feasible, finasteride treatment was started when disease (i.e. hypertrophy/left ventricular dysfunction) was already present in our mice. Despite this late treatment start, finasteride reduced mortality,
effectively inhibited pathological myocardial hypertrophy and fibrosis, left ventricular dilation and systolic as well as diastolic ventricular dysfunction. These results imply an important role of myocardial androgens, more specifically DHT, for the induction of adverse myocardial remodeling. This is supported by data from human weight lifters with anabolic androgenic steroid use, who were shown to develop cardiac hypertrophy as well as systolic and diastolic dysfunction\textsuperscript{28, 29}. Blockade of androgen action by castration (gonadectomy) in male mice prevented pathological hypertrophy, left ventricular dysfunction, fibrosis and dilation in response to TAC induced pressure overload, myocardial infarction, transgenic β2-adrenergic receptor overexpression and lack of the guanylyl cyclase-A\textsuperscript{12, 30-32}. Furthermore, finasteride exerted protective effects on the myocardium in the setting of ischemia/reperfusion injury\textsuperscript{33}. Systemic deletion of the androgen receptor protected male mice from pathological hypertrophy, although - in contradiction to the gonadectomy based studies - it also triggered cardiac dysfunction and fibrosis\textsuperscript{13}. The reason for this discrepancy remains unclear, but could be the consequence of complex secondary effects due to the testicular feminization syndrome observed in these global androgen receptor knock-out mice\textsuperscript{11}. Interestingly, and also striking to us was the fact that finasteride was similar effective to improve ventricular remodeling after TAC in pre-menopausal female compared to male mice. The impact of male sex hormones on the female myocardium is far less well studied. However, in accordance with the fact that androgen receptor expression in the myocardium of females was similarly high compared to males in humans and mice, administration of testosterone or DHT was sufficient to induce adverse ventricular remodeling and hypertrophy in female mice and rats as well as in isolated cardiomyocytes\textsuperscript{7, 12, 34}. Although the total levels of cardiac DHT are very likely to be higher in male than in pre-menopausal
female mice, we found a sharp relative increase in myocardial DHT in female mice in response to TAC.

Despite ample evidence of detrimental androgen effects on the myocardium, data from epidemiologic studies and clinical trials have suggested beneficial systemic effects of testosterone replacement therapy in testosterone deficient men with heart failure. This low dose testosterone replacement therapy particularly improves the exercise capacity, but not left ventricular function, most likely by increasing skeletal muscle mass\textsuperscript{35-37}. In our study, chronic finasteride treatment had no negative effect on skeletal muscle mass. This is in line with a previous study in hypogonadal men, in which low dose testosterone and finasteride treatment were combined, resulting in increased skeletal muscle mass from testosterone, but prevention of deleterious DHT effects on the prostate as a consequence of finasteride administration\textsuperscript{38}.

How did finasteride inhibit cardiac hypertrophy? We demonstrated here that finasteride inhibits activation of important pro-hypertrophic signaling molecules like Akt and its downstream target mTOR in hypertrophied isolated cardiomyocytes \textit{in vitro} as well as in the mouse myocardium after TAC \textit{in vivo}. The action of finasteride on hypertrophy and signaling were dependent on its capability to reduce DHT levels, since addition of DHT in abundance prevented its growth inhibiting properties and ameliorated its impact on mTOR dependent signaling. Finasteride could not inhibit the activation of a constitutive active form of Akt and was no longer effective in reducing hypertrophy in cardiomyocytes expressing activated Akt. In addition, Akt was necessary for the anti-hypertrophic action of finasteride, since inhibition of Akt with a dominant negative construct ablated its effects. Thus, we concluded that modulation of signaling by finasteride was required for its anti-hypertrophic effects and that its impact on cellular signal transduction occurred upstream of Akt. A diminished expression of RCAN1.4
indicated reduced activation of the calcineurin/NFAT pathway in finasteride treated mice after TAC. This certainly contributed to the anti-hypertrophic effects of finasteride, although it could be the consequence of Akt inhibition, which not only results in reduced mTOR activity, but also leads to increased activation of FOXO transcription factors that were shown to inhibit calcineurin\textsuperscript{39}. Testosterone dependent activation of cytosolic signaling molecules like Akt, ERK and mTOR and its contribution to androgen induced hypertrophy has been suggested previously\textsuperscript{40,41}. The impact of androgens on cellular signaling pathways is thought to occur within their “non-genomic” mode of action: instead of operating as a ligand induced transcription factor to regulate gene-expression in the nucleus, androgen receptor binding and activation by DHT occurs at the plasma-membrane, where the assembly of signaling complexes involving the phosphoinositide 3-kinase upstream of Akt is induced in response\textsuperscript{42}.

In conclusion, finasteride effectively inhibits pathological hypertrophy and cardiac dysfunction in mice in response to pressure overload at different stages of the disease or in response to G\textsubscript{aq} over-activation. Finasteride therapy is used as long-term treatment for prostate disease and is generally well tolerated, although rare side effects like sexual dysfunction, depression and high Gleason grade prostate cancer were described\textsuperscript{43}. Another 5-\(\alpha\)-reductase inhibitor, the more potent substance dutasteride (but not finasteride), has been associated with increased risk of heart failure development in one study of patients with prostate disease, although re-analysis of the data by the FDA corrected this effect to be statistically insignificant\textsuperscript{44}. In addition, a recent meta-analysis including more than 18000 patients could not verify any cardiovascular adverse events during dutasteride therapy\textsuperscript{44}. Because finasteride was so effective to reduce cardiac hypertrophy and failure in mice and because of its clinical availability, we suggest that it should be evaluated as a treatment option in heart failure.
Limitation of this study

Although our data – especially the fact that we can reverse the anti-hypertrophic effects of finasteride by DHT in cardiomyocytes – speak against this, we cannot exclude that finasteride may protect the myocardium in vivo independent of its influence on the 5-alpha-reductase and DHT generation. In addition, whether our findings are as relevant in humans as in mice clearly needs further study, also because only a limited number of human myocardial tissue samples were examined here.

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Conflict of Interest Disclosures: TT holds a patent about the use of finasteride to treat cardiac hypertrophy.

References:


Figure Legends:

**Figure 1.** Myocardial expression of the 5α-reductase isoforms (Srd5a1-Srd5a3) as determined by quantitative real-time PCR or Western blot in cardiac hypertrophy and failure. A, Absolute quantification of Srd5a1-3 mRNA expression in the human heart of healthy donors (control, n=9, 5 females), of donors with significant ventricular hypertrophy (n=4, 2 females) and of explanted human hearts with end-stage heart failure (n=23, 6 females). B, Relative quantification of myocardial Srd5a1-3 mRNA in mice two or six weeks (wk) after transverse aortic constriction (TAC) or two weeks after sham surgery (n≥4 mice/group). C, Representative Western blots for Srd5a3 and ACTIN (loading control) from mouse hearts after sham and TAC of different duration. + positive control from mouse testis. D, Quantification of myocardial Srd5a3 protein abundance (n≥6 mice/group). *p<0.05, **p<0.01.

**Figure 2.** Increased myocardial dihydrotestosterone (DHT) levels in hypertrophy. A-B, Mt1a, Odc1 and Axin1 mRNA levels were determined by quantitative real-time PCR as DHT responsive genes in mice three weeks after sham or transverse aortic constriction (TAC) surgery (n=4-6/group), and with or without two weeks of finasteride (Fin) treatment in male (A) or female mice (B). C-E, Western blots for a 25kDa sized protein-DHT complex (C) in neonatal rat cardiomyocytes stimulated with fetal bovine serum (FBS), with or without the addition of finasteride (n=7/group), (D) in the myocardium of male and female mice after TAC and sham surgery as described in (A)-(B), and in healthy (Con) versus hypertrophic human hearts (n≥4/group) (E). ACTIN was used as a loading control. The quantification of the Western blots is shown below. *p<0.05, **p<0.01, ***p<0.001.
Figure 3. Finasteride inhibits hypertrophy in neonatal rat cardiomyocytes (NRCMs). A-B, NRCMs were treated with vehicle (Control), finasteride (Fin), phenylephrine (PE) or fetal bovine serum (FBS). Cardiomyocyte size (A) and the expression of BNP mRNA (B, quantitative real-time PCR) were determined 48 hours after stimulation. C, Cardiomyocyte size of NRCMs as determined 24 hours after stimulation with testosterone (T), dihydrotestosterone (DHT) and finasteride (n=4-8/condition). Representative pictures of anti-troponin stained cardiomyocytes (scale bar, 20μm) are shown in (D). E, Cardiomyocyte size of NRCMs treated as indicated. Fluta denotes flutamide. *p<0.05, **p<0.01, *** p<0.001.

Figure 4. Finasteride inhibits cardiac hypertrophy after transverse aortic constriction (TAC) in male mice. One week after TAC or sham surgery, mice were treated with finasteride (Fin) or left untreated for two weeks. The heart weight/body weight (HW/BW) and the lung weight/BW ratio were determined (n=5-9/group) (A), and the fractional area change was analyzed by echocardiography (n=11-20/group) (B). C, Measurement of ANP, BNP, RCAN1.4 and collagen3 (Col) mRNA in the myocardium of these mice by quantitative real-time PCR (n=4-12/group). D, Quantification of cardiomyocyte cross-sectional area (n=4-5/group). E, Quantification of the myocardial fibrotic area fraction after Sirius Red staining (n=3-6/group). F, Representative pictures (scale bar, 1mm) of Sirius Red stained cardiac sections. G, Serum levels of dihydrotestosterone (DHT) in mice (n=5-10/group). *p<0.05, **p<0.01, *** p<0.001.

Figure 5. Finasteride inhibits cardiac hypertrophy after transverse aortic constriction (TAC) in female mice. One week after TAC or sham surgery, mice were treated with finasteride (Fin) or left untreated for two weeks. The heart weight/body weight (HW/BW) ratio was determined
(n=5-9/group) (A), and the fractional area change was analyzed by echocardiography (n=9-19/group) (B). C, Quantification of cardiomyocyte cross-sectional area (n=3-5/group) and representative pictures of WGA (red) stained sections (scale bar, 50μm) (D). E, The quantification of the myocardial fibrotic area fraction after Sirius Red staining (n=3-4/group) and representative pictures (scale bar, 100μm) are shown (F). *p<0.05, **p<0.01, *** p<0.001.

**Figure 6.** Finasteride (Fin) improves survival and attenuates cardiac dysfunction in long-term pressure overload. A, A time schedule of the experiment is shown. TAC, transverse aortic constriction; echo, echocardiography. B, Kaplan-Meier survival curves of untreated (control) or finasteride treated mice after TAC (n=14 untreated and n=15 finasteride treated animals). C, Fractional area change determined by echocardiography (n=11-26/group). D, dp/dt max, dp/dt min and Tau were measured by Millar catheter in the left ventricle of the indicated mice (n=9-12/group). *p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.

**Figure 7.** Finasteride improves cardiac function in Gq transgenic mice. A, A time schedule of the experiment is shown. WT, wild-type mice; Gq TG, mice with cardiomyocyte specific Gq overexpression; echo, echocardiography. B, Fractional area change (echocardiography) in WT as well as Gq TG (TG) mice with or without finasteride (Fin) treatment. C, Fractional area change in TG mice with or without finasteride treatment at the age of eight or 14 weeks (wk). D, Enddiastolic diameter determined by echocardiography as indicated (n=8-12 mice/group). *p<0.05, **p<0.01, *** p<0.001.
**Figure 8.** Finasteride inhibits cardiomyocyte hypertrophy by interfering with cellular signal transduction. A, Western blots for the indicated proteins from mice three weeks after transverse aortic constriction (TAC) or sham surgery and with or without two weeks of finasteride (Fin) treatment. B, Quantification of the Western blots from (A) (n=3-4/condition). C, Western blots for the indicated proteins from neonatal rat cardiomyocytes (NRCM) treated with fetal bovine serum (FBS), finasteride or dihydrotestosterone (DHT) as indicated. The quantification of p70S6 kinase (p70S6K) activation is shown in (D), (n=5-6/condition). E, Cell size of NRCMs infected with adenoviruses (Ad) Ad.Control (Con) or Ad.caAkt (constitutively active Akt, caAkt) and treated as indicated (n=7/condition). F, Representative Western blots for the indicated proteins of NRCMs after treatment like in (E) and the quantification of Akt and p70S6K activation (G). H, Cell size of NRCMs infected with Ad.Con, an adenovirus encoding for dominant negative Akt (dnAkt) and treated as indicated. *p<0.05, **p<0.01, *** p<0.001, **** p<0.0001.
Figure 1
Figure 3
Figure 4

A

**H/BW (mg/g)**

B

**Lung weight/BW (mg/g)**

C

**ANP mRNA [rel. expression]**

D

**Col mRNA [rel. expression]**

E

**BMP mRNA [rel. expression]**

F

**Cross-sectional area [%]**

G

**DHT [ng/ml]**

Sham Sham+Fin TAC TAC+Fin

Sham Sham+Fin TAC TAC+Fin

Sham Sham+Fin TAC TAC+Fin

Sham Sham+Fin TAC TAC+Fin

Sham Sham+Fin TAC TAC+Fin

Sham Sham+Fin TAC TAC+Fin
Figure 5
Figure 6
Figure 7

(A) Schematic representation of the experimental setup showing the stages of the study:
- WT: Wild Type
- Gqq: Genetic Modification
- TG: Transgenic
- Age: 8 weeks and 14 weeks

(B) Fractional area change [%] for different groups:
- WT
- WT+Fin
- TG
- TG+Fin

(C) Fractional area change [%] for TG groups:
- TG 8wk
- TG 14wk
- No treatment

(D) Enddiastolic diameter [mm] for TG groups:
- TG 8wk
- TG 14wk
- No treatment
- Finasteride
**Figure 8**

A. Western blot analysis of P-AKT, AKT, P-ERK1/2, ERK1/2, P-MTOR, MTOR, P-P70S6K, P70S6K, P-4EBP1, and ACTIN in Sham and TAC-treated samples with and without Fin treatment.

B. Graph showing the percentage change in P-Akt/Akt between Sham and TAC treatments with and without Fin treatment.

C. Western blot analysis of P-AKT, AKT, P-ERK1/2, ERK1/2, P-P70S6K, P70S6K, and ACTIN in Sham and TAC-treated samples with and without Fin treatment.

D. Graph showing the percentage change in P-p70S6K/p70S6K between FBS and DHT treatments with and without Fin treatment.

E. Graph showing the percentage change in Cardiomyocyte size between Ad and Con treatments with and without caAkt treatment.

F. Western blot analysis of P-AKT, AKT, P-ERK1/2, ERK1/2, P-P70S6K, P70S6K, and ACTIN in Ad and Con-treated samples with and without caAkt treatment.

G. Graph showing the percentage change in P-Akt/Akt between Ad and Con treatments with and without caAkt treatment.

H. Graph showing the percentage change in Cardiomyocyte size between Ad and Con treatments with and without dnAkt treatment.
Anti-Androgenic Therapy with Finasteride Attenuates Cardiac Hypertrophy and Left Ventricular Dysfunction

Caroline Zwadlo, Elisa Schmidtmann, Malgorzata Szaroszyk, Badder Kattih, Natali Froese, Hebke Hinz, Jan Dieter Schmitto, Julian Widder, Sandor Batkai, Heike Bähre, Volkhard Kaever, Thomas Thum, Johann Bauersachs and Joerg Heineke

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Expanded Methods

Animals and Animal Procedures

Constriction of the transverse aortic arch (TAC) was performed in 8- to 10 week old C57/Bl6N mice (Charles River) around a 25-gauge needle as previously described\(^1\). Hemizygous transgenic mice expressing the entire coding region of the Gαq protein under the control of the α-myosin heavy chain promoter have been described before and were obtained from the Jackson laboratory\(^2\). Littermate mice not carrying the transgene (wild-type, WT) were used as control. Echocardiography was performed with a linear 30MHz transducer (Visualsonics, Toronto, Canada) in mice that were sedated with 1-1.5% isoflurane and placed on a heating pad to maintain body temperature. LV end-diastolic area (LVEDA) and end-systolic area (LVESA) were recorded. Fractional area change was calculated as \(\frac{(\text{LVEDA}-\text{LVESA})}{\text{LVEDA}} \times 100\). Tail cuff blood pressure was measured in awake mice (BP 2000 Blood Pressure Analysis System, Visitech).

To measure intraventricular pressures, animals were placed on temperature-controlled heating pad and core temperature was maintained at 37°C. A microtip pressure-volume catheter (PVR-1000; Millar Instruments, Houston, TX) was carefully inserted into the left ventricle, cardiac parameters were recorded using Labchart (ADInstrument Ltd. Oxford, UK) to calculate end-systolic and end-diastolic pressure and heart rate, as described\(^3\).

For an assessment of mouse survival after TAC, mice with or without finasteride treatment were followed and inspected daily for 32 days. No mortality was observed in sham operated mice.
Finasteride (Sigma Aldrich, St. Louis, MO) was administered daily by oral gavage as suspension in 0.5% methyl cellulose at a dose of 25mg/kg/day. In order to rule out the unlikely possibility, that the feeding procedure itself exerts beneficial effects after TAC and therefore accounts for the protective effects of finasteride, we analyzed another mouse cohort with the only difference that the mice, which did not receive finasteride were daily fed with NaCl (via an oralgastric tubing). As demonstrated in Supplemental Figure 1, we also found reduced cardiac hypertrophy in the finasteride versus the NaCl treated group. Consequently, in the following experiments we used untreated mice as control for finasteride treatment. At the end of the experiments, mice were weighed and then euthanized. Hearts and lungs were quickly removed from the thoracic cavity. After removal of blood, the heart and lung weights were determined. All animal procedures were approved by our local state authorities.

**Cell culture**

Neonatal rat ventricular cardiomyocytes (NRCMs) were isolated from 1-3 day old Sprague-Dawley rats by Percoll density gradient centrifugation as previously described. On the day after isolation, the cells were switched to serum free media and incubated with phenylephrine (20µM), fetal bovine serum (FBS, 2%), testosterone (1µM), dihydrotestosterone (DHT, 1µM) or vehicle with or without the addition of finasteride (25µM) and/or flutamide (10µM) as indicated. If needed, adenoviral vectors encoding for either β-galactosidase (Ad.Control), constitutive active proteinkinase B/Akt (Ad.caAkt) or dominant negative Akt (Ad.dnAkt) were added to the cells for two hours on the day after isolation, and stimulation with FBS and finasteride was subsequently conducted. Cell size was determined by planimetry after immuno-fluorescence staining for Troponin I (H-
It was determined at least in duplicate cell culture dishes in at least two different cardiomyocyte isolations. Per culture dish, the size of a minimum of 100 cardiomyocytes was analyzed.

**RNA isolation and real-time PCR**

RNA from cultured cardiomyocytes, mouse hearts or human hearts was isolated with the Trifast reagent (Peqlab, Erlangen, Germany). The human heart samples used in this study were in part described previously and obtained after death, explantation or left ventricular assist device implantation with previous institutional review board approval of the Massachusetts General Hospital as well as of the Medizinische Hochschule Hannover\(^5\). Control human heart tissue was derived from victims of motor vehicle accidents, gunshot wounds or from healthy heart organ donors, when the organ was ineligible for transplantation. Hypertrophic human hearts were reported to be taken from patients initially considered as organ donors with known arterial hypertension without any history of congestive heart failure, but with marked cardiac hypertrophy as revealed by echocardiography and/or post-explant examination\(^5\). Failing human heart samples were derived from patients with end-stage heart failure undergoing cardiac transplantation or left ventricular assist device implantation. cDNA was generated from RNA with the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). Quantitative PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and the MX4000 multiplex QPCR system from Stratagene. Gene-expression was normalized to Gapdh mRNA expression or determined with a standard curve established through quantitative PCR of known
amounts of the respective gene product. The PCR primers are listed in the Supplemental Table.

**Western blotting**

Western blot analysis was performed with antibodies to the following proteins (with catalog number and producing company in parenthesis): Akt (9272), phospho-Akt (on Ser473, 9271), phospho-eNOS (on Ser1177, 9571), ERK 1/2 (9102), phospho-ERK1/2 (on Thr202 and Tyr204, 9101), mTOR (on Serine 2448, 2983), phospho-mTOR (5536), phospho-4EBP1 (on Thr 37 and 46, 2855), p70S6 Kinase (9202), phospho-p70S6 Kinase (on Ser371, 9208) (all from Cell Signaling Technology), protein bound dihydrotestosterone (252353, Abbiotec), Srd5a3 (70R-7438, Fitzgerald) and actin (2066, Sigma Aldrich). Densitometry of protein bands was performed using Quantity One (Bio-Rad) software.

**Serum levels of steroid hormones**

We used the AbsoluteIDQ Stero17 kit (Biocrates, Innsbruck, Austria) to assess the concentrations of testosterone, DHT, estradiol and androstendione in mouse serum. The procedure uses a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method and was conducted according to the manufacturer’s instructions as described.

**Histological Analysis**

Transversal frozen sections (7µm in thickness) of the myocardium were generated to measure cardiomyocyte cross-sectional area after staining the cardiomyocyte cell membrane with tetramethyl rhodamine isothiocyanate-conjugated wheat-germ agglutinin...
(WGA, Sigma Aldrich). Fibrosis was quantified with the Sirius Red staining method. Subsequently, the fraction of the fibrotic area (stained in red) from the total myocardial area was determined using Adobe Photoshop Imaging software.

**Statistical Analysis**

All values are presented as means ± SEM. The Mann-Whitney test was used for comparisons between two groups. Differences between three or more groups were analyzed with the Kruskal-Wallis test followed by Dunn´s multiple comparison´s test. Differences between three or more groups in cardiomyocyte cell size in vitro were analyzed by one-way analysis of variance followed by Sidak´s multiple comparisons test after confirmation of Gaussian distribution of values in all experimental groups (by Kolmogorov-Smirnov with Dallal-Wilkinson Lilliefors P value test). Mouse mortality after TAC surgery was analyzed by the log-rank test. A two-tailed P value of less than 0.05 was considered significant. All statistics were calculated with the Graph Pad Prism 6 software.
Supplemental References


## Supplemental Table

### Real-Time PCR Primers, listed from 5´ to 3´ end.

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Supplemental Figure 3
Supplemental Figure Legends

Supplemental Figure 1: One week after TAC (transverse aortic constriction) or sham surgery, mice were treated with finasteride (Fin) or with NaCl for two weeks. Subsequently, the heart weight/body weight (HW/BW) ratio was determined (n=10-13/group). **p<0.01

Supplemental Figure 2: A, Androgen receptor (AR) mRNA expression as determined by quantitative real-time PCR in male or female mice two weeks after transverse aortic constriction (TAC) or sham surgery (n=5-9/group) with or without finasteride (Fin) therapy. B, Testosterone and androstenedione serum levels from mice described under (A). C, Systolic left ventricular pressure and heart rate as determined during Millar catheterization from mice seven weeks after TAC or sham surgery and four weeks of finasteride treatment (n= 9-12/group). D, Systolic (sys) and diastolic (dia) blood pressure as determined by the tail cuff method in male and female Gαq transgenic mice (TG) with or without finasteride treatment (n=3-6/group).

Supplemental Figure 3: A, The mRNA expression of ANP, BNP, RCAN1.4 and collagen3 (Col) as determined by quantitative real-time PCR in the myocardium of female mice three weeks after TAC or sham surgery and two weeks of finasteride (Fin) or no treatment as indicated (n=4-6/group). B, Serum levels of estradiol in mice (n=4 - 9/group). *p<0.05, **p<0.01

Supplemental Figure 4: A, Weights of the gastrocnemius and quadriceps muscles from mice after sham or transverse aortic constriction (TAC) surgery treated with finasteride (Fin) or left untreated as indicated (n=5 -13/group). B, Weights of the same muscles as
in (A) from wild-type (WT) or Gq transgenic (TG) mice at 14 weeks of age (n=8-12 mice/group).

Supplemental Figure 5: A, Quantification of Western blots for the indicated proteins from mice three weeks after transverse aortic constriction (TAC) or sham surgery and with or without two weeks of finasteride treatment (n=3-4/group). B, Quantification of Western blots for the indicated proteins from neonatal rat cardiomyocytes (NRCM) treated as shown (n=3/group). C, Representative pictures of cardiomyocytes infected with a control (Con) adenovirus (Ad) or an adenovirus encoding for an activated form of Akt (caAkt) and treated with finasteride (Fin) or fetal bovine serum (FBS) as indicated (scale bar, 20µm). D, Quantification of Western blots for the indicated proteins from cardiomyocytes treated like in (C) as indicated (n=3/group). E, Cell size of NRCMs infected with adenoviruses (Ad) Ad.Control (Con) or Ad.caAkt (constitutively active Akt, caAkt) and treated with finasteride as indicated (n=7/condition). F, Representative Western blots for the indicated proteins from NRCM treated with adenoviruses Ad.Con or Ad.dnAkt (dominant negative Akt, dnAkt) and FBS as indicated (left side) and its quantification (right side). The dnAkt construct inhibits phosphorylation of eNOS, which is a direct downstream target of Akt. *p<0.05, *** p<0.001