Androgen Receptor-Dependent and Independent Atheroprotection by Testosterone in Male Mice

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The atheroprotective effect of testosterone is thought to require aromatization of testosterone to estradiol, but no study has adequately addressed the role of the androgen receptor (AR), the major pathway for the physiological effects of testosterone. We used AR knockout (ARKO) mice on apolipoprotein E-deficient background to study the role of the AR in testosterone atheroprotection in male mice. Because ARKO mice are testosterone deficient, we sham-operated or orchiectomized (Orx) the mice before puberty, and Orx mice were supplemented with placebo or a physiological testosterone dose. From 8 to 16 wk of age, the mice consumed a high-fat diet. In the aortic root, ARKO mice showed increased atherosclerotic lesion area (+80%, \( P < 0.05 \)). Compared with placebo, testosterone reduced lesion area both in Orx wild-type (WT) mice (by 50%, \( P < 0.001 \)) and ARKO mice (by 24%, \( P < 0.05 \)). However, lesion area was larger in testosterone-supplemented ARKO compared with testosterone-supplemented WT mice (by 57%, \( P < 0.05 \)). In WT mice, testosterone reduced the presence of a necrotic core in the plaque (80% among placebo-treated vs. 12% among testosterone-treated mice; \( P < 0.05 \)), whereas there was no significant effect in ARKO mice (\( P = 0.20 \)). In conclusion, ARKO mice on apolipoprotein E-deficient background display accelerated atherosclerosis. Testosterone treatment reduced atherosclerosis in both WT and ARKO mice. However, the effect on lesion area and complexity was more pronounced in WT than in ARKO mice, and lesion area was larger in ARKO mice even after testosterone supplementation. These results are consistent with an AR-dependent as well as an AR-independent component of testosterone atheroprotection in male mice. (Endocrinology 151: 5428–5437, 2010)

Despite a higher incidence of cardiovascular disease in men compared with women (1, 2), most evidence suggests that androgens, of which testosterone is the most abundant, protect from atherosclerotic disease in men (1, 2). Low serum testosterone generally associates with increased fat mass, an adverse metabolic risk profile, and increased atherosclerosis in men (3–7). Furthermore, despite conflicting data (1, 8), several prospective studies report associations between low testosterone levels and fatal/nonfatal cardiovascular events (9–11). Hence, declining testosterone levels that accompany increasing age may adversely affect both cardiovascular and general aging processes (3). However, no adequately powered studies have yet determined whether testosterone supplemen-
tation protects androgen-deficient and/or elderly or obese men against cardiovascular disease.

Results from animal models support that testosterone confers a beneficial effect on atherogenesis in males. Testosterone treatment reduces atherosclerosis development in both male apolipoprotein E (ApoE)-deficient mice (12, 13) and low-density lipoprotein receptor (LDL-R)-deficient mice (14), as well as other rodent models of atherosclerosis (15–18). Possible mechanisms include beneficial effects on serum lipoprotein levels and antiinflammatory activity by testosterone (1, 2, 12, 14, 19).

Activation of the androgen receptor (AR), which is ubiquitously expressed in tissues, mediates an important part of the physiological effects of testosterone (1, 2). The AR is stimulated either directly by testosterone or by its locally formed metabolite 5α-dihydrotestosterone, a more potent AR agonist than testosterone itself (3). Aromatization of testosterone to estradiol provides an alternative pathway for the effects of testosterone, exerting its effects in turn via signaling pathways distinct from the AR, most importantly via the estrogen receptor (ER)α. Testosterone may also exert effects independently of the classical sex steroid receptors (2).

Two earlier studies addressed putative pathways for the atheroprotective effect of testosterone in male mice. Nathan et al. (14) found that an aromatase inhibitor blocked the effect of testosterone in LDL-R-deficient mice, indirectly indicating that the AR pathway is less important for atheroprotection by testosterone. Nettle-ship et al. (20) studied testicular feminized mice (Tfm) (which carry a naturally occurring AR mutant allele) consuming a cholate-containing diet. They found that exogenous testosterone reduced fatty streak formation in the Tfm mice, indicating an effect of testosterone that is independent of the AR. Importantly, however, Nettle-ship et al. did not treat wild-type (WT) controls with testosterone, and thus could not determine the relative importance of AR-dependent vs. AR-independent pathways. Hence, no previous studies adequately address the role of the AR pathway in the effect of testosterone on atherosclerosis in mice.

Our study aimed to determine the role of the AR in atheroprotection by testosterone in male mice. We studied the atheroprotective effect of testosterone in AR knockout (ARKO) mice and WT littermates on an ApoE-deficient background.

Materials and Methods

Animals

Due to the vital importance of the AR in male fertility, Cre/loxP technology was used to obtain male ARKO mice (21). Mice expressing Cre recombinase ubiquitously under the control of the phosphoglycerate kinase-1 promoter (22) and mice with floxed AR exon 2 (21) were backcrossed to a C57BL/6 background (Charles River Laboratories, Germantown, MD) for at least six generations and then crossed with ApoE-deficient mice (C57BL/6 background, model APOE-M; Taconic Europe A/S, Lille Skensved, Denmark) to obtain a homozygous ApoE-deficient background. Breeding for male mice with ubiquitous knockout of the AR (ARKO) and WT littermates was performed as described previously (21). We assessed AR (21), Cre (21), and apoE (protocol from The Jackson Laboratory, Bar Harbor, ME) genotypes using PCR amplification of genomic DNA. Male sex (i.e. presence of the Y chromosome) was confirmed by the presence of the Zfy gene (21).

Study protocol

All mice were housed in a temperature- and humidity-controlled room with a 0600- to 1800-h light cycle and consumed diet and tap water ad libitum. The mice consumed a soy-free diet (2016; Harlan Teklad, Oxfordshire, UK) after weaning and a high-fat diet (no. 821424, 21% fat from lard, 0.15% cholesterol; Special Diets Services, Essex, UK) beginning at 8 wk of age. All procedures were approved by the Ethics Committee on Animal Care and Use in Gothenburg and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (7th edition, 1996).

At 23–25 d of age, the mice were anesthetized with isoflurane (Baxter Medical AB, Kista, Sweden), either sham operated or bilaterally orchietomized (Orx), and implanted sc with a small slow-release pellet containing placebo or testosterone releasing 25 μg/d during 60 d, corresponding to 2.0 mg/kg at the study start when mean body weight was 12.3 ± 0.3 g (Innovative Research of America, Sarasota, FL). After 50 d, a second 60-d pellet was implanted. A pilot study indicated that treating Orx mice with this dose of testosterone increased the weights of androgen-sensitive organs (e.g. seminal vesicles and ventral prostate) to levels of intact mice (data not shown). The present study included the following groups: WT sham operation + placebo (WT Sham P) (n = 10), WT orchidectomy + placebo (WT Orx P) (n = 11), WT orchidectomy + testosterone (WT Orx T) (n = 8), ARKO sham operation + placebo (ARKO Sham P) (n = 8), ARKO orchidectomy + placebo (ARKO Orx P) (n = 10), and ARKO orchidectomy + testosterone (ARKO Orx T) (n = 12). At 16 wk of age, the mice were fasted for 3–4 h before blood was drawn from the left ventricle, and the circulatory system was perfused with 0.9% saline (pH 7.4) under physiological pressure. The entire aorta (from the heart to the iliac bifurcation) was dissected out, the aortic root was slowly frozen in optimum cutting temperature embedding medium (Sakura Tissue-Tek, Tokyo, Japan), and the rest of the aorta was fixed in 4% paraformaldehyde for subsequent en face evaluation. Tissue from the femoral artery and liver was snap frozen in liquid nitrogen.

Lesion analyses in the aortic root

Serial 10-μm cryosections were cut distally from the aortic root. We stained sections (200, 400, and 600 μm after the appearance of the aortic cusps) with Oil Red O (Sigma-Aldrich, St. Louis, MO) and counterstained them with hematoxylin. For immunohistochemical staining of macrophages, aortic root cryosections (160 μm from the aortic cusps) were incubated with rat antimouse Mac-2 antibody (1:1000; Cedarlane, Hornby, Can-
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Adapted by horseradish peroxidase-conjugated goat antirat IgG secondary antibody (1:1000; GE Healthcare, Buckingham, UK) and visualized with 3,3'-diaminobenzidine (DAB) substrate kit (Dako, Glostrup, Denmark). Staining with Masson's trichrome (240 μm from the aortic cusps) was performed according to the manufacturer's instructions (Accustain Trichrome Stains-Masson from Sigma-Aldrich).

All evaluations of aortic root sections were performed by a blinded observer. We used morphometric analysis (BioPix Software, Gothenburg, Sweden) to determine the size of the atherosclerotic lesions, using two sections from each level (200, 400, and 600 μm from the aortic cusps). Total vessel area, vessel wall area, and lumen area were averaged from two sections (at 200 μm from the aortic cusps). The areas of Mac-2 staining and collagen staining (blue color in Masson's trichrome) were determined using Biopix Software. Lesion complexity (presence/absence of necrotic core and cholesterol crystals) was evaluated in sections stained with Masson's trichrome.

**En face analysis of the aorta**

For *en face* analysis, the aortas were dissected free from connective and adipose tissue, cut open longitudinally, and pinned flat on silicone-coated dishes. The aortas were stained with Sudan IV for lipids, and images were captured. The outline of the aortic surface and atherosclerotic lesions were defined manually by a blinded observer, and lesion areas were computed by an image analysis program (BioPix Software). The extent of atherosclerosis was expressed as the percentage of the aortic surface covered by lesions. Results were calculated for the aortic arch (from the brachiocephalic trunk to the first intercostal arteries), the thoracic aorta (from the first to the last intercostal arteries), and the abdominal aorta (from the last intercostal arteries to the aortic bifurcation), and the whole aorta, respectively.

**Analysis of serum lipids, testosterone, LH, and cytokines**

Serum testosterone was analyzed 30 d after implantation of the second 60-d pellet, and serum collected at the study end was used for other biochemical analyses. We used Infinity reagents (cholesterol no. TR13421 and triglycerides no. TR22421; Thermo Fisher Scientific, Pittsburgh, PA) to analyze total cholesterol and triglycerides in individual serum samples. The distribution of lipids within the plasma lipoprotein fractions was assessed in pooled serum (1 pool per group) by fast-performance liquid chromatography gel filtration using a Superose 6 HR 10/30 column (Pharmacia, Uppsala, Sweden) (23). Serum levels of testosterone and LH were analyzed using RIAs (from MP Biochemicals, Solon, OH, and Immunodiagnostic Systems Ltd., Liege, Belgium, respectively). A cytokine multiplex panel (Mouse Pro-Inflammatory 7-Plex Panel from Meso Scale Discovery, Gaithersburg, MD), including interferon (IFN)γ, IL-1β, IL-6, IL-10, IL-12p70, keratinocyte-derived chemokine, and TNFα, assessed cytokine levels in serum from WT Sham P and ARKO Sham P mice using the electro-chemiluminescence multiplex system Sector 2400 imager (Meso Scale Discovery).

**Blood pressure measurements**

Blood pressure was measured invasively in 16-wk-old WT/ApoE-deficient (n = 10) and ARKO/ApoE-deficient mice (n = 9) after 8 wk on high-fat diet (no. 821424; Special Diets Services).

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**Table 1. Body and organ weights and serum analyses**

<table>
<thead>
<tr>
<th></th>
<th>WT Sham P</th>
<th>WT Orx P</th>
<th>WT Orx T</th>
<th>ARKO Sham P</th>
<th>ARKO Orx P</th>
<th>ARKO Orx T</th>
<th>P (Kruskal-Wallis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (prediet), g</td>
<td>26.9 ± 0.8</td>
<td>21.9 ± 0.4a</td>
<td>24.9 ± 0.4a</td>
<td>22.4 ± 0.8a</td>
<td>22.3 ± 0.3</td>
<td>24.9 ± 0.9a</td>
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<td>Body weight (study end), g</td>
<td>35.2 ± 1.3</td>
<td>29.3 ± 0.9a</td>
<td>31.2 ± 1.4</td>
<td>29.9 ± 1.5a</td>
<td>30.7 ± 0.7</td>
<td>33.6 ± 1.0a</td>
<td>&lt;0.01</td>
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<tr>
<td>Body weight gain (diet period), g</td>
<td>8.3 ± 0.8</td>
<td>7.4 ± 0.6</td>
<td>6.3 ± 1.1</td>
<td>7.4 ± 0.9</td>
<td>8.4 ± 0.5</td>
<td>8.7 ± 0.5</td>
<td>0.29</td>
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<tr>
<td>Testes, mg</td>
<td>188 ± 4</td>
<td>np</td>
<td>np</td>
<td>13 ± 0a</td>
<td>np</td>
<td>np</td>
<td>&lt;0.001</td>
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<tr>
<td>Seminal vesicles, mg</td>
<td>234 ± 10</td>
<td>2 ± 0a</td>
<td>291 ± 29b</td>
<td>np</td>
<td>np</td>
<td>np</td>
<td>&lt;0.001</td>
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<tr>
<td>Ventral prostate, mg</td>
<td>12 ± 1</td>
<td>nd</td>
<td>11 ± 1</td>
<td>np</td>
<td>np</td>
<td>np</td>
<td>0.42</td>
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<tr>
<td>Salivary glands, mg</td>
<td>188 ± 8</td>
<td>86 ± 4a</td>
<td>200 ± 6b</td>
<td>87 ± 3a</td>
<td>86 ± 4</td>
<td>99 ± 3d</td>
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<tr>
<td>Serum testosterone, nmol/liter</td>
<td>1.9 ± 0.8</td>
<td>0.0 ± 0.0a</td>
<td>1.5 ± 0.4b</td>
<td>0.1 ± 0.0a</td>
<td>0.0 ± 0.3c</td>
<td>1.7 ± 0.2d</td>
<td>&lt;0.001</td>
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<tr>
<td>(Serum testosterone, nmol/liter)</td>
<td>(6.6 ± 2.8)</td>
<td>(0.0 ± 0.0a)</td>
<td>(5.4 ± 1.4)</td>
<td>(0.5 ± 0.1a)</td>
<td>(0.0 ± 0.0)</td>
<td>(6.0 ± 0.9)</td>
<td>&lt;0.001</td>
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<tr>
<td>Serum LH, ng/ml</td>
<td>0.19 ± 0.04</td>
<td>2.75 ± 0.40a</td>
<td>0.80 ± 0.24b</td>
<td>3.85 ± 0.62a</td>
<td>3.72 ± 0.38</td>
<td>2.50 ± 0.29d</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/liter</td>
<td>0.94 ± 0.07</td>
<td>0.79 ± 0.06</td>
<td>0.67 ± 0.06</td>
<td>0.95 ± 0.13</td>
<td>0.96 ± 0.11</td>
<td>0.94 ± 0.15</td>
<td>0.22</td>
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Male ARKO and WT mice on ApoE-deficient background were either sham operated (Sham) or Orx before puberty and supplemented with placebo (P) or a physiological testosterone (T) dose. From 8 wk of age, the mice consumed a high-fat diet until the study ended (16 wk of age). n = 7–12/group. Values represent mean ± SEM. np, Not present; nd, not determined.

a P < 0.05 vs. WT Sham P.
b P < 0.05 vs. WT Orx P.
c P < 0.05 vs. ARKO Sham P.
d P < 0.05 vs. ARKO Orx P (Kruskal-Wallis followed by Mann-Whitney U test).

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Female ARKO and WT mice on ApoE-deficient background were either sham operated (Sham) or Orx before puberty and supplemented with placebo (P) or a physiological testosterone (T) dose. From 8 wk of age, the mice consumed a high-fat diet until the study ended (16 wk of age). n = 7–12/group. Values represent mean ± SEM. np, Not present; nd, not determined.

a P < 0.05 vs. WT Sham P.
b P < 0.05 vs. WT Orx P.
c P < 0.05 vs. ARKO Sham P.
d P < 0.05 vs. ARKO Orx P (Kruskal-Wallis followed by Mann-Whitney U test).
The mice were anesthetized with isoflurane (Baxter Medical AB), and a Samba transducer catheter (Samba Sensors, Gothenburg, Sweden) was placed into the left carotid artery for measurements of diastolic, systolic, and mean arterial pressure as well as heart rate. We collected data using a PowerLab data acquisition unit together with LabChart software (ADInstruments, Sydney, Australia), averaged over a period of 2 min, after stabilization of the arterial pressure trace.

RNA isolation and real-time RT-PCR

We extracted total RNA from the femoral artery with RNeasy Fibrous Tissue Mini kit and from the liver using RNeasy Mini kit (QIAGEN, Hilden, Germany). To eliminate DNA contamination, we included a DNase I (QIAGEN) digestion step. First-strand cDNA was synthesized from total RNA using high-capacity cDNA RT kits (Applied Biosystems, Stockholm, Sweden). Real-time RT-PCR analysis was performed with predesigned TaqMan Gene Expression Assays (Applied Biosystems): Mm00433149_m1 (ERα), Mm00598819_m1 (ERβ), Mm00484049_m1 (aromatase), Hs99999901_s1 (18S rRNA), and Mm00607939_m1 (β-actin). Thermal cycling and fluorescence detection was performed with an ABI Prism 7900HT Sequence Detection System and ABI Prism 7900HT SDS Software 2.1 (Applied Biosystems). The thermal cycling conditions were 2 min at 50°C and 10 min at 94.5°C, followed by 40 cycles of 30 s at 97°C and 1 min at 59.7°C. Data were normalized to the reference genes 18S rRNA (femoral artery) and β-actin (liver). We calculated gene expression values using the 2−ΔΔCt method (24).

Statistical analyses

All values represent mean ± SEM. Statistical evaluations were performed with SPSS software (version 15.0; SPSS, Chicago, IL) using the nonparametrical Kruskal-Wallis test (all groups) followed by post hoc testing using Mann-Whitney U test (comparisons of ARKO Sham P vs. WT Sham P; ARKO Orx T vs. WT Orx T; and for the two genotypes, separate comparisons of Orx P vs. Sham P and Orx T vs. Orx P, respectively). Frequencies were tested by χ² test (all groups) and Fisher’s exact test (between two groups). P < 0.05 was considered statistically significant.

Results

As described previously (21), male ARKO mice had female-like external sex organs, cryptorchism, reduced testes weight, and low serum testosterone levels (Table 1). To discriminate between effects attributable to testosterone- and AR-deficiency in the ARKO mice, we Orx or sham operated the mice before puberty and supplemented the Orx mice with testosterone or placebo. In Orx WT mice, testosterone supplementation increased the weights of androgen-sensitive organs (seminal vesicles, ventral prostate, and salivary glands), as well as serum testosterone concentration, to the levels of sham-operated controls and decreased serum levels of LH (Table 1). At both 8 wk (prediet) and 16 wk of age (after 8 wk on high-fat diet), body weight was lower in ARKO vs. WT mice. However, body weight gain during the treatment period was similar in all groups (Table 1).

To examine the importance of the AR for atherosclerosis development, we evaluated the atherosclerotic lesion area both in sections taken from the aortic root (Fig. 1) and in en face preparations of the whole aorta (Fig. 2). Further, lesion complexity and lesion composition were assessed in the aortic root (Fig. 3 and Table 2).

In the aortic root, mean lesion area (Fig. 1A) increased by 80% in ARKO mice compared with WT controls (P < 0.05). Orchiectomy increased the lesion area by 76% (P < 0.05) in WT mice but did not affect atherosclerosis in ARKO mice compared with respective sham-operated control. Compared with placebo, testosterone treatment reduced lesion area by 50% in WT mice (P < 0.001) and 24% in ARKO mice (P < 0.05). The lesion area was significantly larger in testosterone-supplemented ARKO mice compared with tes-
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To assess lesion complexity, we determined the presence/absence of a necrotic core in aortic root sections (Fig. 3). In WT mice, testosterone treatment reduced the frequency of a necrotic core from 80% in placebo-treated mice to 12% in testosterone-treated mice ($P < 0.05$) (Fig. 3C), whereas in ARKO mice, there was no significant effect (67 vs. 33%, $P = 0.20$). Cholesterol clefts were present in most plaques, with no difference between the groups (Table 2). Further, there were no significant differences across the groups in plaque composition, as evaluated by content of collagen, neutral lipids, and macrophages (Table 2).

To search for possible mechanisms for increased atherosclerosis and the reduced effect of testosterone in ARKO mice, we assessed serum lipids at the end of the study. Serum total cholesterol did not differ significantly between ARKO and WT mice ($P = 0.25$) (Fig. 4A). Orchiectomy increased total serum cholesterol by 26% ($P < 0.05$) in WT mice but did not affect ARKO mice (−7%, $P = 0.48$) compared with respective sham-operated control. Compared with placebo, testosterone treatment decreased total cholesterol by 40% ($P < 0.001$) in WT mice but did not affect ARKO mice (−15%, $P = 0.33$). Serum cholesterol tended to be higher in testosterone-supplemented ARKO mice compared with testosterone-supplemented WT mice (36%, $P = 0.079$). Serum triglyceride levels did not differ significantly between the groups (Table 1), and Superoxide 6 chromatography of pooled serum showed similar lipid distribution in lipoprotein fractions among groups (Fig. 4B).

To examine whether increased systemic inflammation is a mechanism for increased atherosclerosis in ARKO mice, we measured serum levels of seven cytokines in ARKO and WT mice. Although serum levels of IL-6, IL-10, IL-1β, keratinocyte-derived chemokine, or IL-12p70 did not differ significantly between ARKO and WT mice, serum levels of TNFα increased in ARKO mice compared with WT mice (30.2 ± 1.5 vs. 26.7 ± 1.0 pg/ml, $P < 0.05$). Furthermore, levels of IFNγ tended to increase (12.8 ± 2.5 vs. 10.1 ± 3.4 pg/ml, $P = 0.083$) in the ARKO mice.

To examine a possible difference in blood pressure, we assessed blood pressure invasively in ARKO and WT mice. However, neither systolic (ARKO vs. WT: 132 ± 6 vs. 130 ± 5 mm Hg, $P = 1.0$), diastolic (93 ± 4 vs. 91 ± 2 mm Hg, $P = 0.93$), mean arterial blood pressure (106 ± 5 vs. 104 ± 3 mm Hg, $P = 0.93$), nor heart rate (459 ± 20 vs. 447 ± 20 beats/min, $P = 0.53$) differed between groups.
To examine the possibility that decreased ERα, ERβ, or aromatase expression may explain reduced atheroprotection by testosterone in ARKO mice, we assessed mRNA expression of ERα, ERβ, and aromatase in the femoral artery and liver. There were no significant differences in ERα mRNA expression in the femoral artery or ERβ mRNA in the liver (Table 3). In the liver, ERα mRNA expression increased in ARKO compared with WT mice, and orchiectomy increased ERα mRNA in the liver in WT but not ARKO mice. We detected no ERβ mRNA in the femoral artery and no aromatase mRNA expression in the femoral artery or the liver by quantitative PCR.

Discussion

Our study demonstrates that the AR participates in the atheroprotective effect of testosterone in male ApoE-deficient mice. In analyses of the aortic root, as well as in en face evaluation of the whole aorta, the atheroprotective effect of testosterone in ARKO mice was less than half the effect observed in WT controls, demonstrating that the AR pathway is a major pathway for the effect of testosterone on atherosclerosis in this model. Further, an AR-independent action of testosterone was demonstrated in the aortic root and in en face evaluation of the thoracic aorta.

Ours is the first study that specifically addresses the relative role of the AR in atheroprotection in mice. Nettleship et al. (20) found that exogenous testosterone normalized fatty streak formation in the AR mutant Tfm mice fed a cholate-containing diet and suggested that this finding indicates that most of the effect of testosterone is AR independent. However, the study treated only Tfm and not WT controls with testosterone, and thus could not determine the relative importance of AR-dependent vs. AR-independent pathways. In accordance with our results, treatment with flutamide, an AR blocker, inhibited most of the protective effect of testosterone on atherosclerotic plaque area in cholesterol-fed rabbits (18). Further, the AR agonist 5α-dihydrotestosterone reduced atherosclerotic plaque area in the brachiocephalic artery of male ApoE-deficient mice (13). Our present finding also concurs with the important role of the AR pathway in many of the physiological effects of testosterone (25).

Although our study indicates that a large part of the effect of testosterone is AR dependent, testosterone reduced atherosclerotic lesion area in ARKO mice, consistent with an AR-independent component of the testosterone effect. Aromatization of testosterone to estradiol may mediate an AR-independent action of testosterone by activating, in turn, the ERs. ERα has been implicated in atheroprotection by estradiol, whereas ERβ reportedly is less important (26). Nathan et al. (14) found that an aromatase inhibitor completely blocked the effect of testosterone on atherosclerosis in LDL-R-deficient mice, indicating that conversion to estradiol exerted the entire effect of testosterone. In comparison, Nettleship et al. (20) found that an aromatase inhibitor blocked only 20% of the effect of testosterone on fatty streak formation in Tfm mice.

Because of the result suggesting that aromatization plays a crucial role in the effect of testosterone on atherosclerosis in LDL-R-deficient mice (14), we examined the possibility that the reduced atheroprotection by testosterone in ApoE-deficient ARKO mice may be explained by decreased ERα or aromatase expression in the ARKO model. However, ERα mRNA did not decrease in the fem-
oral artery or liver of ARKO mice, hence we found no support for altered ERα expression accounting for the attenuated effect of testosterone in ARKO mice. Indeed, in line with previous findings (27, 28), ERα mRNA increased in the liver of WT mice after orchietomy as well as in ARKO compared with WT mice. We detected aromatase mRNA expression in neither the femoral artery nor the liver, confirming the low level of aromatase expression in extragonadal tissues of mice (29). Hence, we cannot exclude a possible regulation of local aromatase expression in the ARKO model. On the other hand, given the complex interplay between estrogens, androgens, and their receptors (30), it cannot be excluded that aromatase inhibitors alter AR expression. Furthermore, the role of aromatization may differ between mouse models of atherosclerosis. Importantly, our present results are compatible with an AR-independent pathway of testosterone, including the aromatization pathway, which comprises about half of the atheroprotective effect of testosterone in male mice.

Due to the normally occurring large variation in serum testosterone levels among WT males (31), evaluating the weight of androgen-sensitive male reproductive organs provides an important validation of the testosterone dose used in experimental studies. Our testosterone treatment increased both serum testosterone levels and the weight of androgen-sensitive organs to the levels of intact WT controls, confirming that our treatment regimen (slow-release pellets, 25 μg/d) was physiological. Furthermore, our testosterone treatment decreased the degree of aortic root atherosclerosis in Orx WT mice to the level of intact WT mice. In contrast, the testosterone treatment appeared slightly supraphysiological in its effect on serum cholesterol at the study end, although LH levels at the same time point do not support that the testosterone levels were too high. It is likely that the testosterone dose used influences the relative importance of the different pathways for the testosterone effect (2). Treating LDL-R-deficient mice with testosterone (139 μg/d) using slow-release pellets from the same manufacturer, Nathan et al. (14) decreased atherosclerosis in Orx mice to the levels of intact controls but did not report the weight of androgen-sensitive organs and reported serum testosterone only as a single value from pooled samples. It is conceivable that the higher testosterone dose used by Nathan et al. (5.6 times higher than
much interest has focused on demonstrating the general efficacy and long-term safety of testosterone therapy (3, 35), although few large controlled studies demonstrated the magnitude of the effects, this requires further study.

Increased atherosclerosis. However, given the small magnitude of the effects, this requires further study.

In conclusion, ARKO mice on ApoE-deficient background display accelerated atherosclerosis. Testosterone treatment reduced atherosclerosis in both WT and ARKO mice. However, the effect on lesion area and complexity was more pronounced in WT than in ARKO mice. These results are consistent with an AR-dependent as well as an AR-independent component of testosterone atheroprotection in male mice.

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<table>
<thead>
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<th>Table 3. mRNA expression in femoral artery and liver</th>
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<tr>
<td>Femoral artery</td>
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<td>ERα mRNA, arbitrary units</td>
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<tr>
<td>Liver</td>
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<td>ERβ mRNA, arbitrary units</td>
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</table>

The findings reported here may have important clinical implications. Although few large controlled studies demonstrate the general efficacy and long-term safety of testosterone therapy (3, 35), much interest has focused on testosterone supplementation to elderly men, as evidenced by a 20-fold increase in testosterone sales in the United States during the 1990s (3, 10). The androgen-AR system is extensively manipulated during treatment of prostate cancer in men, and our results are potentially important for ongoing efforts to limit the cardiovascular side effects of different treatment regimens (36–38). Although considerably less well developed than selective ER modulators, compounds that activate or inhibit the AR in a tissue-specific way (selective AR modulators (SARMs)) are now garnering interest in the goal to achieve beneficial effects on bone and muscle mass without adversely affecting, e.g., the prostate (39). Our present finding should be taken into account in the design and evaluation of SARMs. Furthermore, SARMs aimed primarily at prevention or treatment of cardiovascular disease require further examination.
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