Androgen-Induced Progression of Arterial Calcification in Apolipoprotein E-Null Mice Is Uncoupled from Plaque Growth and Lipid Levels

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Arterial calcification has prognostic significance for cardiovascular outcomes, but its pathogenesis remains unclear. Calcification increases with age, but its prevalence in men suggests hormonal influence. In this study we analyzed the effect of exogenous androgens on calcification of advanced atherosclerotic lesions in the arterial tree of gonadally intact 34-wk-old male and female apolipoprotein E-null mice. Testosterone (T) increased calcification 3- to 4-fold ($P < 0.05$) in lesions of the innominate artery and aortic sinus. A nonaromatizable androgen, dihydrotestosterone, also increased lesion calcification in the innominate artery (2.4-fold, $P < 0.05$) but not the aortic sinus. The androgen-induced effects were independent of sex and occurred despite corresponding reductions in plaque area, the latter correlating inversely with increased serum high-density lipoprotein cholesterol levels. Androgen-induced calcification in the innominate artery was observed with up-regulation of local androgen receptor (AR) expression in response to T and dihydrotestosterone for both males and females but neither androgen influenced innominate artery estrogen receptor (ER)-α or -β expression in either sex. Conversely, T-induced calcification in the aortic sinus was associated with down-regulation of ERα but not ERβ expression in both sexes, whereas androgen-induced AR expression was increased in female but decreased in male mice. This study demonstrates for the first time that calcification of advanced atherosclerotic lesions is an androgen-sensitive process and postulates potential roles for both AR- and ER-mediated pathways in androgen-induced vascular calcification. We demonstrate a novel direct link between vascular calcification and the major male hormone, T, uncoupled from conventional relationships with plaque growth and lipid levels. **(*Endocrinology* 150: 841–848, 2009)**

The increasing use of androgens in both older men and women to alleviate features of aging and the continued abuse of anabolic androgens in sports (1) poses important questions about the cardiovascular safety of exogenous androgens. Whereas male sex is a well-known risk factor for cardiovascular disease (CVD) (2), the relationship between endogenous and exogenous, pharmacological, and physiological doses of androgens and CVD remains ambiguous. The hypothesis that male hormones are proatherogenic (3) has received support from recent *in vitro* studies showing androgens augment early steps in atherogenesis in a sex-specific manner (4–6). However, in animal models of early stages of atherosclerosis, testosterone (T) enhances plaque development in females but reduces it in males, via aromatization of T to estradiol (7–9). The overall effects of androgens in accelerating the atherosclerotic process may involve not only influence on lesion establishment but also on the progression and morphology of advanced lesions. Such effects might become manifest as early adverse effects of androgen therapy in older men with unrecognized underlying CVD, in a manner analogous to the early excess adverse cardiovascular effects seen with estrogen therapy in recent prospective trials in women (10).

Calcification is an important morphological and prognostic feature of advanced arterial plaques. Arterial calcification is a strong predictor of coronary events in humans (11–13) and increases complications associated with angioplasty or stent place-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2008-0760 Received May 20, 2008. Accepted October 9, 2008.
First Published Online October 16, 2008

Abbreviations: Apo, Apolipoprotein; AR, androgen receptor; CBFA1, core binding factor; COLI, type I collagen; CVD, cardiovascular disease; DHT, dihydrotestosterone; ER, estrogen receptor; HDL, high-density lipoprotein; H&E, hematoxylin and eosin; PLSD, protected least significant difference; SOX, Sry-type high-mobility-group box transcription factor; T, testosterone.

ment (14, 15). Plaque calcification is recognized as an active rather than passive degenerative process (16), sharing many similarities with bone mineralization (17). Despite the common features, pathogenesis of vascular calcification remains poorly understood. The paradox that, in older age bone mineralization declines whereas vascular calcification increases, is perplexing, given their mechanistic overlap, and defining their common regulatory factors is important to better understand these complex mechanisms.

Arterial calcification increases with age, but its higher prevalence in men suggests possible hormonal influence (18). Sex hormones and androgens, in particular, play a major regulatory role in bone formation in the young and bone homeostasis in the adult. Androgens have significant effects on bone formation via regulation of both osteoblast (bone forming) and osteoclast (bone resorbing) cell types (17). Despite the identification of these cellular phenotypes within advanced calcifying lesions (19, 20), the effects of androgens on the calcification of established atherosclerotic lesions have not been reported. In the present study, we examined the effects of exogenous androgen treatment on vascular calcification using a gonadally intact apolipoprotein (Apo) E-null mouse model of atherosclerosis.

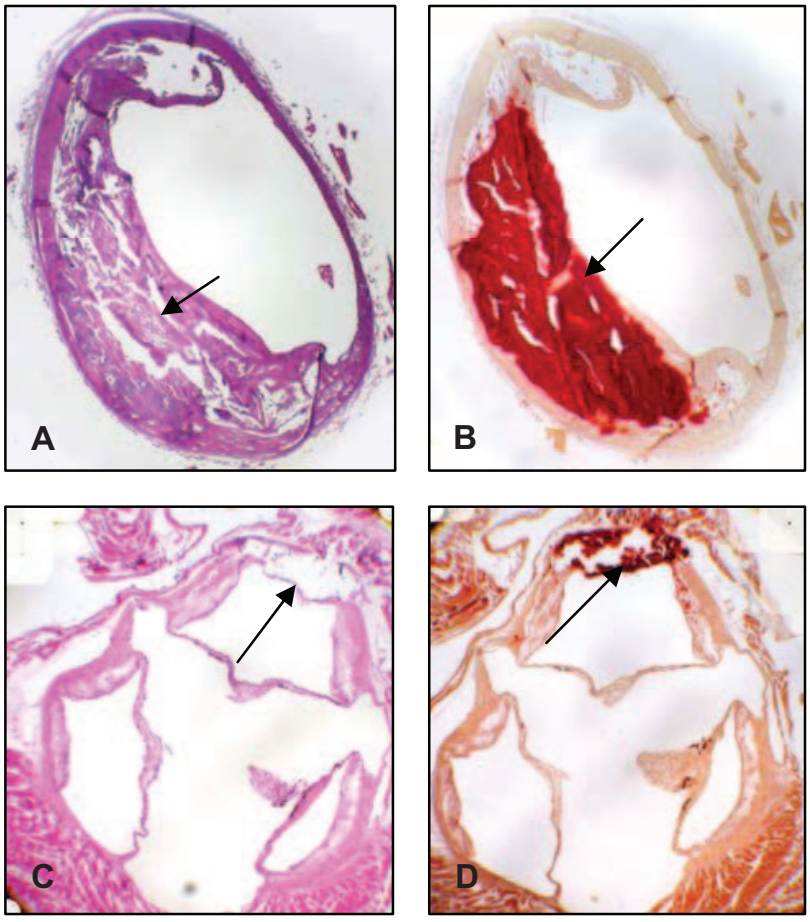

FIG. 1. Comparison of calcium staining in H&E and Alizarin Red S-stained arterial sections. Consecutive sections from the innominate artery (A and B) or aortic sinus (C and D) were stained with H&E (A and C) or Alizarin Red S (B and D). Calcified regions were identified in H&E-stained sections by their *dark purple*, birefringent staining (*arrow*, A), which often dissected during sectioning (*arrow*, C). Calcified regions stained a *dark red color* with Alizarin Red S (*arrows*, B and D).

Materials and Methods

Animals

ApoE-null mice were obtained from the Animal Resources Centre (Perth, Australia) to establish a breeding colony maintained in the animal facility at the Heart Research Institute. Mice were provided with standard approved housing conditions and had free access to water and a regular chow diet. All experiments were approved by the Animal Welfare Committee of the Sydney South West Area Health Services.

Groups of male and female mice aged 33–34 wk were randomly divided into control and treatment groups (n = 10 mice/group). Androgens were administered as 1 cm SILASTIC brand implants (Dow Corning, Midland, MI) filled with either crystalline T or dihydrotestosterone (DHT) implanted subdermally at the back of the neck under methoxyflurane anesthesia as described previously (21). Control mice were untreated. After 8 wk total body weights were recorded. Mice were anesthetized with 1 U pentobarbitone (6 mg /ml; BOMAC Laboratories, Sydney, Australia) per gram body weight and killed by cardiac exsanguination with 0.5–1 ml of blood taken to store serum for measurement of cholesterol and T concentrations. The arterial tree was fixed by perfusion through the left ventricle at physiological pressure using warmed (37 C) PBS plus heparin (10 U/ml) for 4 min followed by 4% (wt/vol) paraformaldehyde (pH 7.5) for 6 min. The arterial tree was dissected out and fixed in 4% (wt/vol) paraformaldehyde overnight at room temperature. The tissue was then washed in PBS and stored in 70% (vol/vol) ethanol. Seminal vesicles were removed and weighed as an *in vivo* index of androgen bioactivity.

Dissection and definition of lesion area

The aortic sinus and innominate artery were separated from the rest of the arterial tree before dehydration and paraffin embedding to allow cross-sectioning of each tissue. A series of six duplicate cross-sections (5 μm) were taken from the aortic sinus for estimation of plaque area, beginning with the appearance of the first valve leaflet. Duplicate sections were taken every 100 μm thereafter until a series of six slides in total had been collected. A set of six duplicate cross-sections were taken from the innominate artery every 110 μm beginning at the region proximal to the arch for determination of plaque area. Sections were stained with hematoxylin and eosin (H&E) and plaque area determined as the combined number of pixels in all six sections by an observer blinded to the treatment group using Adobe Photoshop 7.0 (San Jose, CA). In the innominate artery, plaque area was considered to lie between the internal elastic lamina and the endothelium. In the aortic sinus, because of extensive medial involvement, the estimated plaque area included both the intimal and medial layers. Because plaque area in 34-wk-old mice was not determined, it could not be conclusively established whether lesions were stable or progressing at the commencement of treatment; hence, changes are referred to in terms of plaque size rather than plaque growth.

Calcified areas were identified in H&E-stained sections by their basophilic, birefringent staining (dark purple, crystalline) and quantified in the same serial sections and in the same manner used to determine total plaque area. These areas were confirmed as calcium containing by staining consecutive sections with Alizarin Red S (Fig. 1). It should be noted that total calcified areas may have been underestimated due to the preservation methods used. Tissues were fixed, dehydrated, and embedded in the same way for an equivalent length of time to minimize any differential loss between tissue specimens. Sections (5 μm) taken in the intervening regions were used for immunohistochemical analysis.

TABLE 1. Mean body, seminal vesicle weight, and serum T and lipid concentrations in androgen-treated ApoE-null mice at the time the animals were killed

Group	Body weight (g)	T (nmol/liter)	Seminal vesicle weight (g)	Total cholesterol (mmol/liter)	HDL-C (mmol/liter)	ApoAI (μg/dl)
Male control	29.6 ± 0.5	13.2 ± 5.6	0.42 ± 0.02	29.2 ± 1.9	0.53 ± 0.05	96.6 ± 5.3
Male + T	31.9 ± 0.6[a]	25.4 ± 2.9[a]	0.69 ± 0.05[b]	27.0 ± 2.1	0.50 ± 0.06	95.9 ± 7.3
Male + DHT	31.5 ± 0.9[a]	ND	0.55 ± 0.05[a]	32.6 ± 4.8	0.79 ± 0.04[b]	123 ± 6.9[a]
Female control	23.5 ± 0.4[c]	1.36 ± 0.2		25.3 ± 2.0	0.31 ± 0.03[d]	71.5 ± 3.3[c]
Female + T	25.0 ± 0.4[b]	26.8 ± 6.2[d]		24.0 ± 2.6	0.57 ± 0.09[b]	79.8 ± 2.0[a]
Female + DHT	25.4 ± 0.5[b]	ND		30.0 ± 2.5	0.54 ± 0.03[b]	93.1 ± 7.3[b]

Mean values ± SEM are presented and the level of significance for the differences between treatment groups, and corresponding sex-specific controls were determined using one-way ANOVA and Fisher's PLSD *post hoc* test. Significant differences between male and female control groups and T concentrations in control and T-treated female animals were calculated using the Mann-Whitney *U* test. Serum T was not measured in DHT-treated mice due to high cross-reactivity of DHT in the nonchromatographic T immunoassay. ND, Not determined.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$.

[d] $P < 0.01$.

Immunohistochemical analysis

Sections were stained with antiaromatase antibody (Abcam, Cambridge, UK; no. ab18995-50), antiandrogen receptor (AR) antibody (Upstate, Lake Placid, NY; no. 06-680), and the following antibodies obtained from Santa Cruz Biotechnologies (Santa Cruz, CA): antiestrogen receptor (ER)-α (sc-7207); anti-ERβ (sc-8974); anti-type I collagen (COLI; sc-25974); anticore binding factor α1 (CBFA1; sc-10758); and anti Sry-type high-mobility-group box transcription factor (SOX) 9 (sc-20095). Control sections were incubated with rabbit (Dako, Glostrup, Denmark; no. X09360) or goat IgG (Santa Cruz Biotechnologies; sc-2028) to eliminate false-positive staining. Protein expression was detected after tyramide signal amplification as per the manufacturer's instructions (PerkinElmer Life Sciences, Norwalk, CT; no. SAT700). Color development was achieved using diaminobenzidine.

Sections were digitally captured using a BH-2 Olympus microscope (Tokyo, Japan). Relative staining intensity in coded sections was determined in a blinded fashion by two observers using ImageJ version 1.37 (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/:). The mean integrated density values (measured in pixels) were determined independently on representative sections using three fields of view from four mice per treatment group and analyzed using one-way ANOVA. Final data are shown relative to the male control. Representative immunostained images are shown in the on-line data supplement (Figs. I-VIII, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Blood analysis

Terminal blood obtained by cardiac puncture was stored on ice before separating serum by centrifugation (4 C, 5000 rpm, 5 min) for storage at −20 C until analysis in a single batch. Total serum cholesterol and high-density lipoprotein (HDL)-cholesterol were determined by colorimetric assay (Roche Diagnostics, Mannheim, Germany). HDL levels were determined after ApoB particles/low-density lipoprotein were precipitated by polyethylene glycol for 20 min. ApoA-I was determined by ELISA using plates coated with goat antimouse ApoA-I and rabbit antimouse ApoA-I secondary antibody (Biodesign, Saco, ME). Blood T concentration was measured in organic solvent (ethyl acetate-hexane) extracts of serum without chromatography by immunofluorometric assay as described previously (21).

Statistical analysis

To stabilize the variance and normalize the data, calcium and plaque area (converted to square millimeters) were subject to square root transformation for statistical analyses using StatView 5.0.1 (SAS Institute Inc., Cary, NC). The statistical significance of differences was calculated using one-way ANOVA and Fisher's protected least significant difference (PLSD) *post hoc* test. Where specified, the Mann-Whitney *U* test was used to compare nonparametric data. $P < 0.05$ was considered statistically significant. The significance of categorical factors (sex, site, androgen treatment) as predictors for the presence of calcification in lesions was analyzed by logistic regression.

Results

Mouse weights, serum cholesterol, and T estimations

Body and seminal vesicle weight as well as serum T and lipid concentrations at the time the animals were killed are shown in Table 1. T implantation increased serum T approximately 2-fold ($P < 0.05$) in males and 20-fold ($P < 0.01$) in females to reach male levels. Serum T was not measured in DHT-treated mice due to high cross-reactivity of DHT in the nonchromatographic T immunoassay.

Neither T nor DHT treatment had any significant effect on total cholesterol levels in either male or female mice. HDL cholesterol levels were significantly lower in female compared with male controls ($P < 0.01$) and were increased 1.8-fold ($P < 0.01$) in T-treated and 1.7-fold ($P < 0.001$) in DHT-treated females to reach male levels. Whereas there was no change in HDL cholesterol levels in T-treated male mice, DHT treatment further increased male HDL cholesterol levels (1.5-fold, $P < 0.01$).

Androgens do not advance plaque area in older mice

T treatment did not alter plaque size in male mice in either region studied (Fig. 2, A and B). In female mice, T treatment decreased both innominate (1.5-fold, $P < 0.001$, n = 6) and aortic sinus (1.2-fold, $P < 0.05$, n = 7) plaque size. DHT treatment decreased plaque size 1.2-fold in the aortic sinus in both genders ($P < 0.05$, n = 7). In the innominate artery, DHT showed a similar trend; however, this did not reach statistical significance.

Androgens increase vascular calcification

Lesions showed morphological features characteristic of advanced human atherosclerotic lesions with the most striking dif-

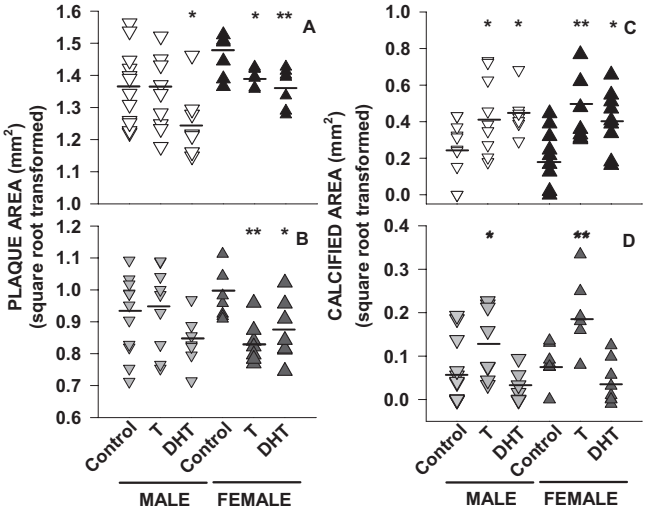

FIG. 2. Plaque and calcified area in androgen-treated mice. Plaque area (A and B) and calcified area (C and D) were determined in the innominate artery (A and C) and aortic sinus (B and D) of male and female ApoE-null mice treated with T or DHT. Data were measured in square millimeters and normalized by square root transformation (transformed data shown). *Bars* represent the data mean. One-way ANOVA was used to determined significant differences on square root transformed data with Fisher's PLSD *post hoc* analysis (*, $P < 0.05$;**, $P < 0.01$ *vs.* respective sex-specific control).

ference observed between treatment groups in the degree of intimal calcification. Table 2 shows the frequency (absence/presence) of calcification in each treatment group. Logistic regression analysis showed a significant effect for T treatment ($P < 0.001$) after adjustment for site and sex.

Calcified area was quantified in stained sections from both regions (Fig. 2, C and D). T treatment increased mean calcification 2.7-fold in the innominate artery of both male ($P < 0.05$, n = 9) and female ($P < 0.05$, n = 7) mice as well as in the aortic sinus of male (4.1-fold, $P < 0.05$, n = 7) and female (4.4-fold, $P < 0.05$, n = 7) mice. DHT treatment also increased calcification 2.4-fold in the innominate artery of male ($P < 0.05$, n = 7) and female ($P < 0.01$, n = 10) mice, whereas in the aortic sinus, DHT treatment did not change calcification significantly.

Linear regression analysis found no significant correlations between plaque area and calcified area in any treatment group (not shown). As a percentage of plaque area, calcified area was more extensive in the innominate artery compared with the aortic sinus in both untreated male (innominate: 10.0 ± 2.9% *vs.* sinus: 0.5 ± 0.2%, $P < 0.01$) and female mice (innominate: 7.5 ± 3.0% *vs.* sinus: 0.4 ± 0.1%; $P < 0.01$) (Mann Whitney *U* test) (data not shown).

TABLE 2. Frequency of lesion calcification

Treatment group	Number of animals exhibiting calcification (%)[a]	
	Innominate artery	Aortic sinus
Male control	8 of 10 (80%)	7 of 12 (58%)
Male + T	9 of 9 (100%)	9 of 9 (100%)
Male + DHT	7 of 7 (100%)	4 of 7 (57%)
Female control	8 of 9 (89%)	6 of 7 (86%)
Female + T	7 of 7 (100%)	6 of 6 (100%)
Female + DHT	9 of 9 (100%)	5 of 8 (63%)

T treatment increased calcification significantly ($P < 0.001$), regardless of site or sex.

[a] Significance was analyzed by logistic regression.

Site-specific effect of androgens on AR expression

Androgen regulation of AR expression varied with arterial site (Fig. 3, A and B). In the innominate artery, AR expression in the intimal region increased in both T (6-fold, male, $P < 0.05$; 3-fold, female, $P < 0.01$) and DHT-treated (9-fold, male, $P < 0.001$; 3-fold, female, $P < 0.01$) mice (Fig. 3A) independent of sex. In the aortic sinus, androgen effects on AR expression were sex specific (Fig. 3B). In male mice, both T and DHT treatment decreased intimal AR expression (T: 3.3-fold, $P < 0.001$; DHT: 1.7-fold, $P < 0.01$), whereas in female mice, T and DHT treatment increased AR expression (1.5-fold, $P < 0.05$) relative to untreated controls.

Sex-independent effect of androgens on ERα expression

ERα expression in the intimal region of the aortic sinus (Fig. 3D) was decreased by T treatment in both male and female mice (2.2-fold, female, $P < 0.01$; 3.3-fold, male, $P < 0.05$). In contrast, DHT-treatment increased ERα expression in male (2-fold, $P < 0.001$) but not female mice. In the innominate artery, similar trends did not reach statistical significance (Fig. 3C). No difference in staining intensity of ERβ was observed between treatment groups in either region studied or either sex (Fig. 3, E and F). Aromatase was expressed in each region but decreased only in response to T in the aortic sinus of male mice (1.5-fold, $P < 0.05$) (Fig. 3, G and H).

Coexpression of AR and osteogenic markers in mineralized regions

Because cells expressing high levels of AR in the innominate artery coincided with areas of mineralization, they were examined for expression of markers consistent with osteoblast or chondrocyte-like cell development. Adjacent sections taken from the innominate artery of both male and female mice were stained with antibodies against the osteoblast-specific transcription factor CBFA1, the matrix component COLI, and the chondrocyte-specific transcription factor SOX9 (Fig. 4, A–L). Strong nuclear AR staining colocalized with expression of these markers within Alizarin S-stained calcified regions. Cells in the surrounding non-mineralized regions that did not show strong AR expression also lacked expression of either CBFA1 or SOX9. In the aortic sinus, CBFA1 expression was localized to small focal regions of mineralization (Fig. 4, M–O). AR and ER expression reflected expression in unidentified cell types not expressing osteogenic markers.

Discussion

In this study, exogenous T and DHT treatment had striking effects to increase the extent of intimal calcification of advanced atherosclerotic lesions in both male and female ApoE-null mice and unexpectedly occurred despite simultaneous decreases in plaque size and increases in serum HDL cholesterol. The ability

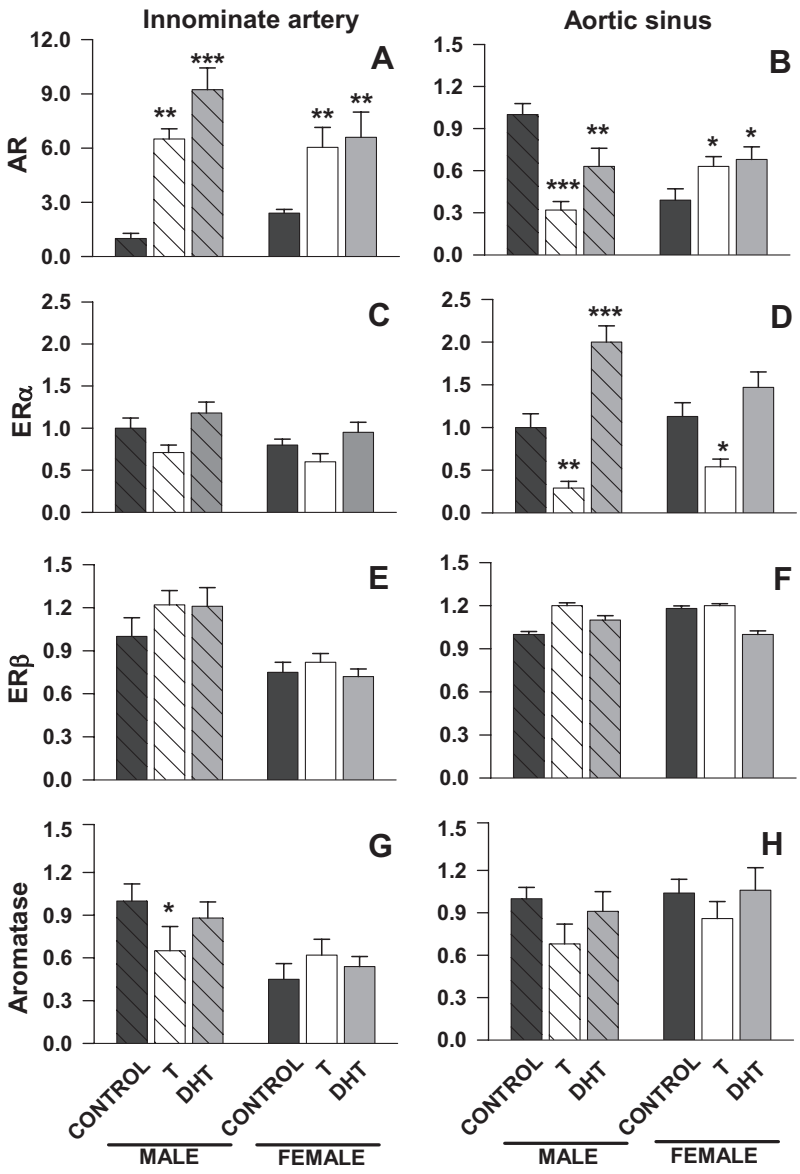

FIG. 3. Protein expression of sex hormone receptors and aromatase in lesions of androgen-treated mice. Relative levels of AR (A and B), ERα (C and D), ERβ (E and F), and aromatase (G and H) in the innominate artery (A, C, E, and G) and aortic sinus (B, D, F, and H) of T and DHT-treated ApoE-null mice. Mean staining intensity was determined within intimal plaque regions in stained sections using ImageJ as described in *Materials and Methods*. Data are presented as mean ± SEM (n = 4) and were normalized relative to male controls (value of 1). Data (in pixels) were compared between groups of same sex using one-way ANOVA and Fisher's PLSD *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

calcification. Estrogen-replacement studies predominantly show estrogens reduce coronary calcification (26, 27), consistent with the finding that low serum estradiol levels in women are correlated with increased arterial calcification (28).

Consistent with our findings, an association between long-term anabolic steroid abuse and early vascular calcification has been noted in men (29). Similarly, in women with polycystic ovary syndrome, elevated endogenous androgen levels are associated with increased vascular calcification (30) and high-dose T administration in postmenopausal women has been associated with increased atherosclerosis as measured by detection of calcified deposits (31). These findings were not disassociated, however, from concomitant increases in plaque growth obscuring a more direct relationship between androgens and calcification. Our study is the first to show in an animal model a direct link between the major male hormone, T, and calcification in the vasculature.

One limitation of this study is that we show effects at supraphysiological rather than physiological levels of androgens, given the doses administered. These findings are congruent with the findings in women exposed to excessive endogenous or exogenous T (30, 31). There is no evidence so far of any direct association between calcification and physiological levels of T in men; however, our findings suggest further studies into this possible relationship are warranted.

It was striking in the present study that calcification was not associated with increases in plaque size, to which it has been traditionally linked (12). Larger plaques are generally more complex and therefore more likely to be calcified, and any lag in lesion formation would be expected to delay formation of advanced lesion characteristics (32). Androgen treatment in this study resulted in either no effect on plaque area (T treated males) or a decrease in plaque area (T treated females and both male and female DHT treated mice). Decreases in lesion area were associated with androgen effects to increase serum HDL cholesterol, in agreement with other studies showing an inverse relationship between HDL cholesterol and atherosclerosis (33, 34). The systemic modulation of HDL cholesterol by exogenous androgen treatment seems to preclude any clear association between local sex hormone receptor expression and plaque size. Coronary plaque area has previously been associated positively with intimal ERβ and inversely with medial AR expression in men; however, this was in the absence of any exogenous hormone treatment (35).

The uncoupling of calcification from plaque size in this mouse model appears to be a fortuitous effect of androgens to promote an antiatherogenic lipid profile in mice, an effect previously observed (34), in contrast to that promoted in humans (29, 36, 37).

of DHT to fully replicate T effects in the innominate artery suggests that these androgenic effects are mediated via the AR; however, more complex mechanisms appear to involve aromatization and ER-mediated pathways in the aortic sinus.

Although there is anecdotal evidence showing men have more coronary calcification than women (18), suggesting hormonal involvement, it has remained unclear whether this discrepancy reflects estrogen protection in women, androgen aggravation in men, or a combination of both. *In vitro*, estradiol treatment shows variable effects on calcification with both inhibition (22) and stimulation of vascular smooth muscle cell calcification (23) reported. In animal models, estradiol treatment has shown either null (24) or beneficial (25) effects on plaque progression and

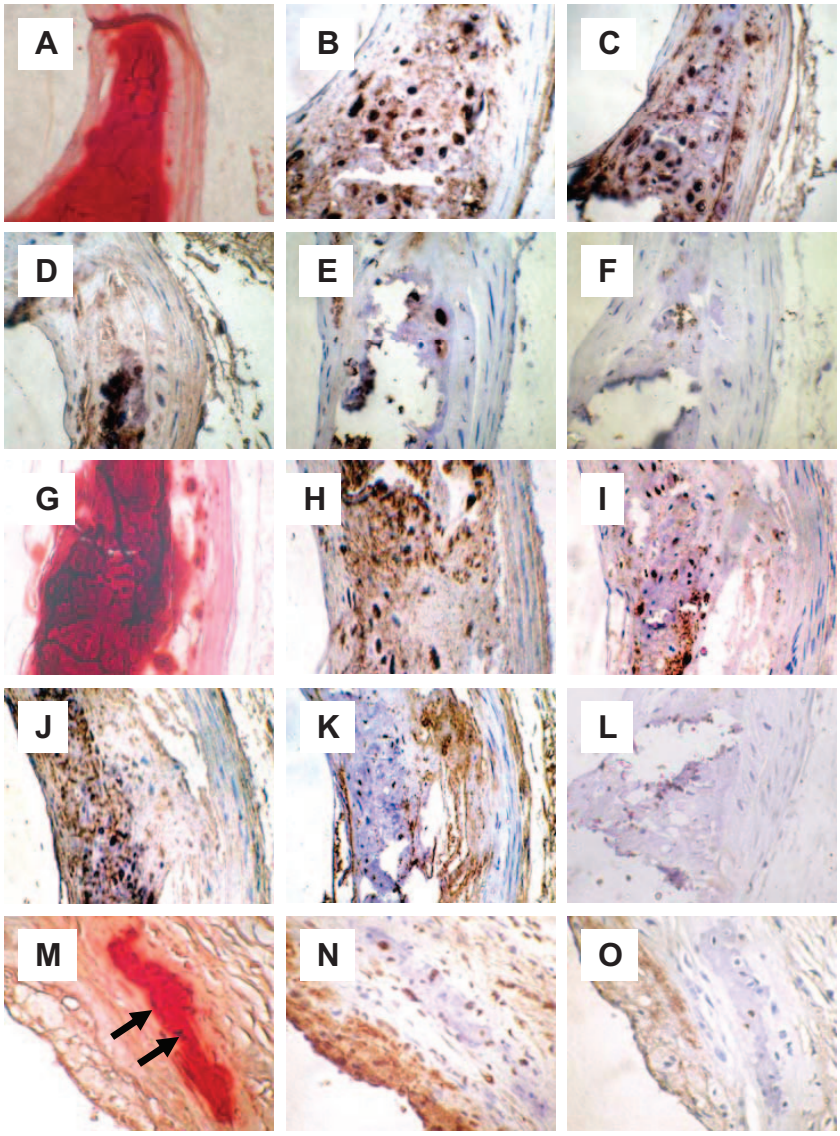

FIG. 4. Colocalization of high AR expression with bone-specific markers in lesions of androgen-treated mice. Adjacent sections were taken from the innominate artery of a representative male (A–F) and female (G–L) ApoE-null mice treated with T and stained to show colocalization of AR with regions of calcification and expression of bone-specific markers (A–L). Calcified areas were identified using Alizarin Red S staining (A and G). Protein expression was examined using primary antibodies against AR (B and H), CBFA1 (C and I), COLI (D and J), and SOX9 (E and K). CBFA1 was expressed in nuclei of cells within small localized regions in the aortic sinus (N) of T-treated mice associated with mineralization (M, *black arrows*) but not in regions lacking calcification. Biotinylated secondary antibodies were detected using diaminobenzidine. Rabbit IgG isotype control was used to eliminate false-positive staining in each region (F, L, and O). Sections were counterstained with hematoxylin. Magnification, ×40.

Uncoupling of plaque growth and calcification has been reported previously in both pre- and postmenopausal women, in whom independent correlations were found between estrogen status and coronary calcium, plaque burden, and plaque to calcium ratio (38). These observations reinforce the view that calcification is an active, hormone-sensitive process commandeering conventional osteogenic mechanisms, not simply a passive degenerative consequence of plaque development (16).

Because the nonaromatizable androgen, DHT, increased plaque calcification in the innominate artery in a congruent manner to T, the androgen effects appear to be direct effects manifest through the AR rather than indirect effects on ERs after aromatization of T to estradiol. Consistent with this hypothesis, local AR expression in the innominate artery was dramatically increased with calcification in response to both T and DHT. AR expression in the vasculature is hormonally regulated, but regulation can be positive or negative depending on cell and tissue type (39). *In vitro* studies show consistently that AR is up-regulated in osteoblasts or osteosarcoma cells by androgens (40–42) and that this mediates increased androgen responsiveness (43), including direct stimulation of mineralization (41, 44, 45). Similarly, *in vivo*, osteoblasts undergoing active mineralization at the edge of bone also express high levels of AR (46). A direct association exists therefore between high AR expression in osteoblast-like cells and increased mineralization. Localization of up-regulated AR expression with chondrogenic (SOX9) and osteogenic (CBFA1) markers in calcified regions in this study suggests that androgens may have a similar effect on mineralization in the innominate artery through an AR-mediated pathway.

The discrepant effect of DHT on calcification in the aortic sinus in both male and female animals was surprising and more difficult to reconcile; however, site-specific variations in lesion formation and progression in response to various systemic treatments have been reported previously (47). The divergence of the effects of T and DHT on calcification in the aortic sinus is more consistent with a pathway involving aromatization and ERs rather than a mechanism mediated via AR. In agreement with this, the gender-specific pattern of AR expression was not consistent with the gender-independent pattern of calcification in this region, as was found in the innominate artery. Instead the increase in calcification in T-treated animals was associated with a simultaneous decrease in ERα expression. Loss of ERα has been associated previously with calcification in one man lacking a functional ERα who had impaired vascular function and early coronary artery calcification (48). In contrast, another study found that ERβ, but not ERα, was regulated by estrogen therapy in pre- and postmenopausal women and was associated with both atherosclerosis and coronary calcification (49). ERβ expression was not influenced by T or DHT in this study; however, it remains possible that any influence changes in ERα expression may exert on calcification could be a result of modulation of the ERα to ERβ ratio, as noted in other settings when both are expressed in the same cell (50).

In contrast to the present findings, Liu *et al.* recently reported no association between coronary artery calcification in men and AR, ERα, or ERβ expression (35). The exclusion from that study

of subjects with existing coronary artery disease and correspondingly high levels of calcification may have led to underestimation of any association between calcification and receptor expression in more advanced, calcified lesions.

The site-specific androgen effects on calcification and sex hormone receptor expression may be due to differences in lesion complexity driven by the variability in hydrodynamic forces acting at each site (47) and consequently the phenotype of resident cells. A study by Balkan *et al.* (42) showed that potent androgens (mibolerone and DHT) had variable effects on calcification and AR expression of preosteoblastic cells *in vitro*, depending on the stage at which hormone was added, suggesting some dual stimulatory/inhibitory effect of androgens on early osteoblastic development. In the present study, the area calcified as a percentage of total plaque area was far smaller in the aortic sinus of both male and female mice and as a result fewer cells expressed markers of osteoblast differentiation. We postulate that T may influence earlier steps in osteoblast functional formation after aromatization and modulation of ERα activity in the lesions of this region.

Further work is required to define any role of ERs in vascular calcification. The correlation of expression patterns with calcification does not demonstrate causality but provides an important first step in determining the underlying mechanisms by which androgens exert their effects. The findings suggest a potential role for both AR- and ER-mediated pathways in the development of vascular calcification in response to androgens. The present study shows the first direct link between pharmacological androgen use and vascular calcification and provides the basis for better understanding the role of androgens in the pathogenesis of advanced calcified atherosclerotic plaque, which has become highly relevant given the increasing pharmacological use of androgens in society.

Acknowledgments

The expert assistance of Mark Jimenez in providing hormone implants and for serum testosterone measurements and Assoc. Prof. Jeffrey Cohn for assistance with lipid analysis are gratefully acknowledged.

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This work was supported by research grants from the National Heart Foundation of Australia and the Bruce and Joy Reid Foundation. L.M. was supported by a University Postgraduate Award cofunded by the Discipline of Medicine, University of Sydney, and the Heart Research Institute.

Disclosure Statement: The authors have nothing to disclose.

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