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Androgen Receptor-dependent Transactivation of Growth Arrest-specific Gene 6 Mediates Inhibitory Effects of Testosterone on Vascular Calcification*[S]

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Recent epidemiological studies have found that androgen deficiency is associated with a higher incidence of cardiovascular disease in men. However, little is known about the mechanism underlying the cardioprotective effects of androgens. Here we show the inhibitory effects of testosterone on vascular calcification and a critical role of androgen receptor (AR)-dependent transactivation of growth arrest-specific gene 6 (Gas6), a key regulator of inorganic phosphate (P_i)-induced calcification of vascular smooth muscle cells (VSMC). Testosterone and nonaromatizable androgen dihydrotestosterone inhibited P_i-induced calcification of human aortic VSMC in a concentration-dependent manner. Androgen inhibited P_i-induced VSMC apoptosis, an essential process for VSMC calcification. The effects on VSMC calcification were mediated by restoration of P_i-induced down-regulation of Gas6 expression and a subsequent reduction of Akt phosphorylation. These effects of androgen were blocked by an AR antagonist, flutamide, but not by an estrogen receptor antagonist, ICI 182,780. We then explored the mechanistic role of the AR in Gas6 expression and found an abundant expression of AR predominantly in the nucleus of VSMC and two consensus ARE sequences in the Gas6 promoter region. Dihydrotestosterone stimulated Gas6 promoter activity, and this effect was abrogated by flutamide and by AR siRNA. Site-specific mutation revealed that the proximal ARE was essential for androgen-dependent transactivation of Gas6. Furthermore, chromatin immunoprecipitation assays demonstrated ligand-dependent binding of the AR to the proximal ARE of Gas6. These results indicate that AR signaling directly regulates Gas6 transcription, which leads to inhibition of vascular calcification, and provides a mechanistic insight into the cardioprotective action of androgens.

Recent clinical studies have suggested that a low plasma testosterone level is associated with advanced atherosclerosis and is independently related to cardiovascular disease and death (1–5). Many but not all animal studies have also shown inhibitory effects of androgens on experimental atherosclerosis and vascular remodeling (6–8). Also, several clinical studies indicate that the testosterone level is inversely related to vascular calcification, a significant feature of vascular pathology (9). However, the mechanism underlying the vasoprotective effects of androgens is poorly understood.

Most of the actions of testosterone, particularly of nonaromatizable dihydrotestosterone (DHT),[2] are mediated by the androgen receptor (AR) (10, 11). In the nucleus the AR activates transcription by binding to androgen-response elements (AREs) in the promoter and enhancer regions of target genes (12). It further has been reported that AR is expressed in all layers of the arterial wall (13) and is involved in vascular disease (14, 15). However, the precise mechanism such as the signaling and molecular target of the AR has not been addressed.

We recently reported that growth arrest-specific gene 6 (Gas6) is a key molecule regulating calcification of vascular smooth muscle cells (VSMC) through the survival signal transduction mediated by phosphatidylinositol 3-OH kinase/Akt phosphorylation (16, 17). Gas6 is a member of the vitamin K-dependent protein family and is a secreted protein that harbors a γ-carboxylglutamic acid-rich domain and four epidermal growth factor-like repeats (18). In the present study we showed transcriptional activity of the AR in VSMC and an inhibitory effect of androgens on inorganic phosphate (P_i)-induced VSMC calcification. The inhibitory effect of androgens on VSMC calcification was attributable to restoration of the Gas6-mediated survival pathway. Furthermore, we found that the AR directly binds to the ARE in the Gas6 promoter region and transactivates the Gas6 gene.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: DHT, dihydrotestosterone; AR, androgen receptor; ARE, androgen-response element; Gas6, growth arrest-specific gene 6; VSMC, vascular smooth muscle cells; HASMC, human aortic smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; siRNA, small interfering RNA; Act D, actinomycin D; ChIP, chromatin immunoprecipitation; luc, luciferase.

EXPERIMENTAL PROCEDURES

Cell Culture—Human aortic smooth muscle cells (HASMC) derived from a 32-year-old man were purchased from Clonetics. HASMC were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. HASMC were used up to passage 8 for the experiments. In preliminary experiments HASMC were cultured in a calcifying condition of 2.6 mM P_i in DMEM without phenol red with 15% dextran-charcoal-stripped serum to remove steroids from the culture medium. This condition, however, induced marked apoptosis and an increase in calcification (4.7 ± 0.5-fold). Consequently, we performed all experiments in DMEM with 15% complete serum-supplemented medium. Human prostate cancer LNCaP and PC-3 cell lines were maintained in RPMI (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Materials—Testosterone, DHT, 17β-estradiol, and flutamide, an AR antagonist, were purchased from Sigma. ICI 182,780 was obtained from TOCRIS. These materials were dissolved in absolute ethanol and added to the cultures from a 1000-fold-concentrated stock. Control cultures received similar amounts of ethanol only. Final ethanol concentration did not exceed 0.1% (v/v).

Promoter Reporter Construct—The 1925-bp (−1827/+99) and 1070-bp (−971/+99) Gas6 promoter corresponding to the Gas6 promoter sequences were generated by PCR from human genomic DNA with the appropriate sets of primers. These inserts were cloned into a pGL3 basic vector (Promega). The pGL3-Gas6-ARE mutant construct was made by performing site-directed mutagenesis (Stratagene) with the appropriate primer pairs: AA82CC, 5′-CTGAGAATGGCAAGCCCTCCATTAACTCTC-3′ (forward primer) and 5′-GAGAGTTAATGGAGGGCTTGCCATTCTCAG-3′ (reverse primer); AA1281TT, 5′-CCAAGACAAGAGCCAGTTAGTCTTGGTCTCTGAAG-3′ (forward primer) and 5′-CTTCAGAGACCAAGACTAACTGGCTCTTGTCTTGG-3′ (reverse primer); CT 1292 GA, 5′-GAGCCAGAAAGTCTTGGTGACTGAAGACAAGCACAATG-3′ (forward primer) and 5′-CATTGTGCTTGTCTTCAGTCACCAAGACTTTCTGGCTC-3′ (reverse primer). The constructs were verified by sequencing. The construct of ARE-luciferase (luc) was described previously (19).

Luciferase Assay—HASMC were seeded in 12-well plates at a density of 7×10^4 cells/well and were transiently transfected with 0.8 μg of ARE-luc construct or Gas6-luc construct using Lipofectamine 2000 (Invitrogen) according to the procedure recommended by the manufacturer. The next day the cells were treated with testosterone, DHT, or ethanol vehicle for an additional 24 h. Aliquots of 20 μl of cleared lysate were assayed with a luciferase assay kit from Promega. Luciferase activity was normalized to that of vehicle-treated cells and adjusted to the cell protein content.

Small Interfering RNA—Two small interfering RNAs (siRNAs) were designed to target human Gas6 (GenBank™ accession no. NM_000820) using siRNA design software (Dharmacon). The sequences of Gas6 siRNA were 5′-GUGACGAGGGCUUUGCGUA-3′ and 5′-GGAGAAGGCUUGCCGAGAU-3′. To evaluate the effect of Gas6 siRNA on calcium deposition, both of two siRNA were transfected when HASMC had reached 80∼90% confluence and then transfected every time the medium was changed (every 2 days) up to 6 days. AR (GenBank™ accession no. NM_001011645) was knocked down with two siRNAs to evaluate the role of the AR in androgen-stimulated Gas6 transcription activity. The sequences of AR siRNA were 5′-GAGCGUGGACUUUCCGGAA-3′ and 5′-UCAAGGAACUCGAUCGUAU-3′ (Dharmacon). In HASMC, 6 h after transfection of the Gas6-luc construct, the two AR siRNAs or control siRNA (100 nM) was transfected using transfection reagent (Upstate Biotechnology). The next day DHT or ethanol vehicle was added for an additional 24 h, then luciferase assay was performed. The efficiency of siRNA was validated by immunoblotting the cell lysates at 48 h after transfection.

RNA Extraction, Real-time PCR, and mRNA Stability Analysis—Total RNA was prepared using an RNeasy RNA extraction kit (Qiagen); 3 μg of total RNA from each of triplicate samples were reverse-transcribed into cDNA using an Omniscript first-strand synthesis system (Qiagen) according to the manufacturer's protocol. Assays for each sample were performed in triplicate using a 7300 real-time PCR system (Applied Biosystems). Then 5 μl of the cDNA sample was amplified by PCR in a total reaction volume of 50 μl using SYBR Green master mix (Applied Biosystems) and 500 nM concentrations of the forward 5′-GCCTTTCAGGTCTTCGAGGAG-3′ and reverse 5′-GTCAGGCAGGTTTTGCACG-3′ primers specific to Gas6. Amplification conditions were 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Data were analyzed by $2^{-\Delta\Delta Ct}$ method. The relative expression values of all mRNAs were normalized to the β-actin mRNA level (forward 5′-CTGGAACGGTGAAGGTGACA-3′ and reverse 5′-AAGGGACTTCCTGTAACAATGC A-3′).

To examine Gas6 mRNA stability, HASMC were incubated with actinomycin D (Act D, 5 μg/ml) in the presence or absence of 2.6 mM P_i or DHT treatment (12 h). Total RNA was extracted at 0, 3, and 6 h after Act D treatment, and the decrease in mRNA expression was determined by real-time PCR analysis as described above. The RNA degradation curve was obtained by setting the maximum mRNA expression at 0 h before Act D treatment as 100%.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, HASMC were treated with DHT or ethanol vehicle for 12 h and cross-linked with 1% formaldehyde for 10 min at room temperature. After the cells were collected, nuclei were prepared by incubating the cells in SDS lysis buffer (50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS). Chromatin was sheered by sonication to an average size of 500∼1000 base pairs and diluted 10-fold with dilution buffer. Immunoprecipitation was performed using a polyclonal AR antibody (Santa Cruz Biotechnology), polyclonal acetyl-histone H3 antibody (Millipore), monoclonal p300 antibody (Millipore), and polyclonal rabbit IgG antibody (Santa Cruz Biotechnology). PCR amplification of the Gas6 promoter region spanning the ARE was performed using the following

primers: proximal ARE (5′-GGATGCTGGGCTAACTGC-3′) and 5′-GCAACATTGTGCTTGTCTTCA-3′); distal ARE (5′-CAGGCAGAGGCTAGAGATGC-3′ and 5′-CAGCAGCCC-ATGGATAAACT-3′). In all cases PCR was performed with serial dilutions of the input and various numbers of cycles (25~40 cycles) to ensure that amplification was maintained in the linear range.

Quantification of Calcification—For P_i-induced calcification, P_i (a mixed solution of Na_2HPO_4 and NaH_2PO_4 whose pH was adjusted to 7.4) was added to serum-supplemented DMEM to a final concentration of 2.6 mM. Calcium deposition was evaluated by the *o*-cresolphthalein complexone method (C-Test; WAKO) and von Kossa staining, as previously described (20).

Determination of Apoptosis—To examine the effect of androgens on P_i-induced apoptosis, androgens were added simultaneously to switch the medium of HASMC to medium containing 2.6 mM P_i. Apoptosis was detected by measuring DNA fragmentation with a cell-death detection ELISA^{plus} kit (Roche Applied Science) according to the manufacturer's instructions.

Immunoblotting and Immunofluorescent Analysis—To examine the location of the AR protein, HASMC were separated into cytoplasmic and nuclear fractions using a nuclear extract kit (Active Motif). Nuclear and cytoplasmic fractions (20~30 μg) were applied to SDS-polyacrylamide gels under reducing conditions and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis was performed using anti-AR polyclonal antibody (Santa Cruz Biotechnology). The effect of androgens on expression of Gas6, phospho-Akt and Akt were examined, as described previously (20). HASMC were grown in 15% fetal bovine serum in DMEM on 2-well chamber slides and fixed in 4% paraformaldehyde for 10 min, and for the AR assay they were incubated with rabbit anti-AR antibody at a 1:250 dilution. Detection of the AR was performed with a 1:100 dilution of fluorescein isothiocyanate-conjugated anti-rabbit antibody (Invitrogen). After several washes, the slides were counterstained with 4′,6-diamidino-2-phenylindole.

Statistical Analysis—All values are presented as the mean ± S.E. Statistical comparisons were made by analysis of variance followed by Fisher's test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Functional Androgen Receptor Expressed in the VSMC Nucleus—To investigate the action of androgens in VSMC, we first examined whether the AR is expressed in VSMC. In comparison with AR-positive (LNCaP) and AR-negative (PC-3) prostate cancer cells, we found that AR was endogenously expressed in HASMC (Fig. 1*A*). To determine the location of its expression, we separated the cytoplasmic and nuclear fractions of HASMC. AR was expressed mainly in the nucleus (Fig. 1*B*). These results were confirmed by immunofluorescence of the AR (Fig. 1*C*). Next, to examine whether the AR expressed in VSMC is functional, we transfected the ARE-luc construct into HASMC. Androgens (testosterone and DHT) increased luciferase activity by 2~2.5-fold, whereas 17β-estradiol did not affect its activity. Furthermore, androgen-stimulated ARE activity was abrogated by flutamide, an AR antagonist (Fig. 1*D*). Taken together these results indicate that the AR expressed in the nucleus of VSMC participated in androgen-mediated regulation of the ARE.

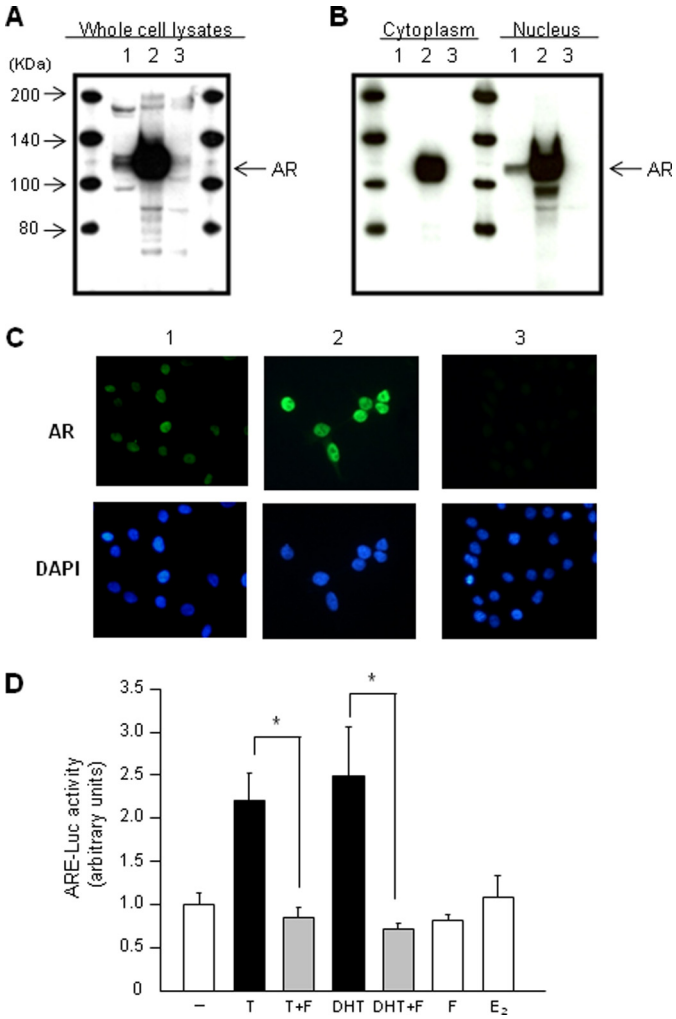


FIGURE 1. **Expression of the functional the AR in HASMC.** Endogenous expression of the AR in HASMC (*lane 1*) was examined in whole cell lysates (*A*) and cytoplasmic and nuclear fractions (*B*) compared with that in human prostate cancer cell lines, LNCaP (AR-positive; *lane 2*) and PC-3 (AR-negative; *lane 3*). *C*, AR expression was also detected by immunofluorescent staining (*green*). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*, *blue*). *D*, HASMC were transiently transfected with 0.8 μg of the ARE-luciferase construct. Twenty-four hours after transfection, androgens (testosterone (*T*) and DHT, 100 nM), 17β-estradiol (*E2*, 100 nM), and flutamide (*F*; 10 μM) were added, and the cells were incubated for an additional 24 h. Relative promoter activities are expressed as the mean ± S.E. of quadruplicate samples. Similar results were obtained from four independent experiments. *, $p < 0.05$ by Fisher's test.

Androgens Inhibit P_i-induced VSMC Calcification by Restoration of Gas6-mediated Survival Pathway—To investigate the role of the AR in VSMC, we examined the effects of androgens on vascular calcification, a critical and advanced phenotype of atherosclerosis. In the model of P_i-induced calcification (16), calcium deposition was significantly suppressed by both androgens in a concentration-dependent manner (Fig. 2, *A* and *B*). We then examined whether the effect of androgens was mediated by the AR. The effect of androgens was clearly abolished by flutamide but not by ICI 182,780, an estrogen receptor antagonist (Fig. 2*C*). Similar effects on calcification were confirmed by von Kossa staining (Fig. 2*D*).

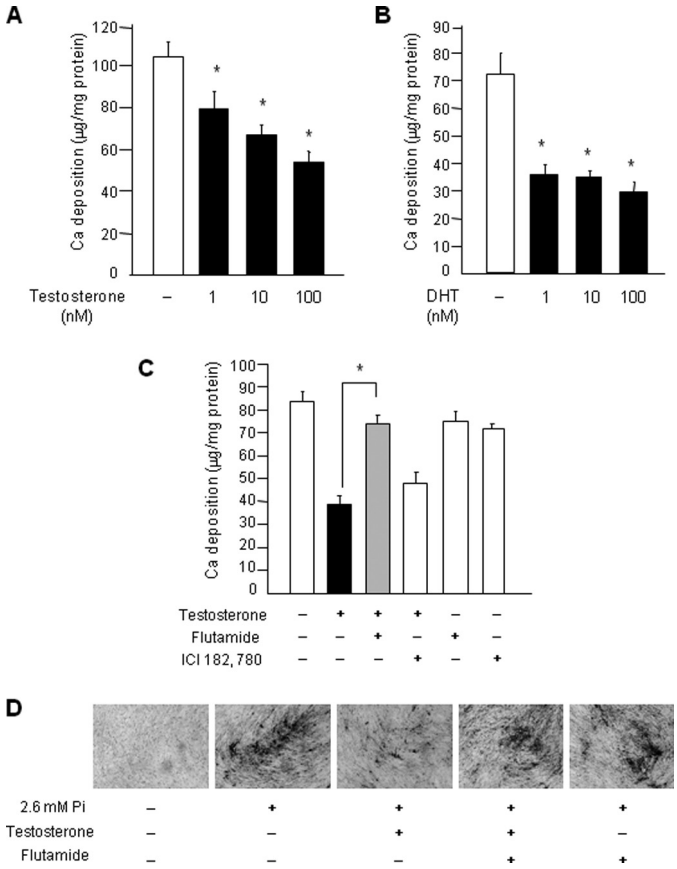


FIGURE 2. **Androgens prevent P_i-induced calcification via the AR.** HASMC were cultured with the indicated concentrations of androgens (testosterone (*A*) and DHT (*B*)) in the presence of 2.6 mM P_i for 6 days. Calcium deposition was measured by the *o*-cresolphthalein complexone method and normalized by cell protein content. *, $p < 0.05$ *versus* androgens (−) by Fisher's test. HASMC were cultured with flutamide (10 μM) or ICI 182,780 (10 μM) in the presence or absence of testosterone (100 nM) with 2.6 mM P_i treatment. On day 6 calcium deposition was measured (*C*) and was evaluated at the light microscopic level with von Kossa staining (*D*). All values of calcium deposition are presented as the mean ± S.E. of quintuplicate samples. Similar results were obtained from three independent experiments. *, $p < 0.05$ by Fisher's test.

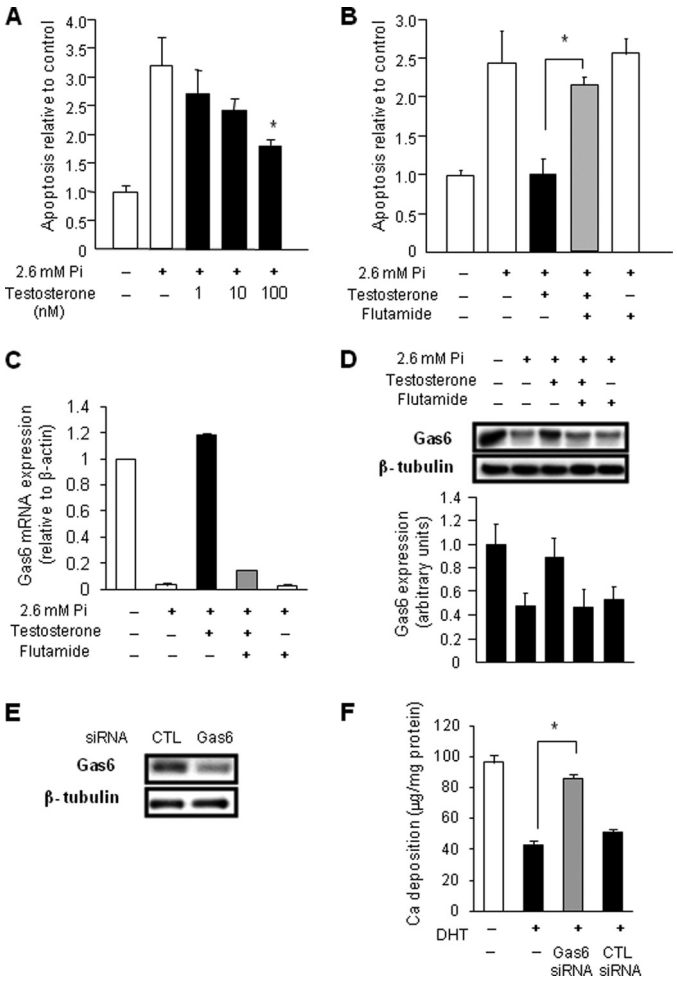


FIGURE 3. **Androgens inhibit P_i-induced apoptosis and restore Gas6-mediated survival pathway.** *A*, HASMC were cultured with the indicated concentrations of testosterone in the presence of 2.6 mM P_i for 6 days. A quantitative index of apoptosis, determined by DNA fragmentation enzyme-linked immunosorbent assay, is presented as the value relative to that without P_i treatment. *, $p < 0.05$ *versus* 2.6 mM P_i, testosterone (−) by Fisher's test. *B*, HASMC were treated with testosterone (100 nM), or flutamide (10 μM) in the presence of 2.6 mM P_i for 6 days. *C* and *D*, on day 6, RNA and cell lysates were harvested and analyzed for Gas6 mRNA and protein levels by real-time PCR (*C*) and immunoblotting (*D*), respectively. β-Actin mRNA and β-tubulin protein levels were also measured as loading control. The average results of three separate measurements of mRNA are shown. The *panel* shows a representative blot, and *bar graphs* show quantitative analyses of three independent immunoblotting experiments. *E*, HASMC were transfected with two Gas6 or control siRNA (100 nM). Gas6 protein was efficiently decreased by two siRNAs targeting Gas6 at 48 h after transfection. *CTL*, control. *F*, for measurement of calcium deposition, HASMC were transfected with 100 nM Gas6 siRNA and nonspecific (*CTL*) siRNA and incubated with DHT (100 nM) and 2.6 mM P_i for 6 days. All values of apoptosis and calcium deposition are presented as the mean ± S.E. of triplicate samples. Similar results were obtained from three independent experiments. *, $p < 0.05$ by Fisher's test.

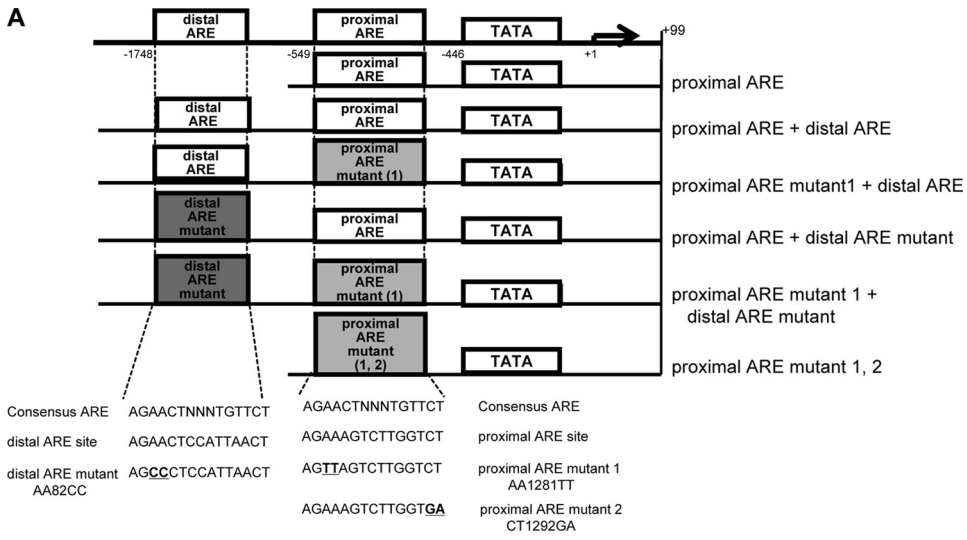
Because apoptosis is a crucial and initiating event in P_i-induced VSMC calcification (16, 17), we examined whether androgens inhibit P_i-induced apoptosis. Furthermore, in our recent study apoptosis induced by P_i has been shown to be associated with inhibition of Gas6 expression and secretion (16, 17). Androgens, at concentrations exerting an inhibitory effect on calcification, significantly reduced P_i-induced apoptosis, as quantified by analysis of cytoplasmic histone-associated DNA fragments (Fig. 3*A*). Flutamide significantly abrogated the inhibitory effect of androgens on apoptosis in HASMC (Fig. 3*B*). We further examined the effect of androgens on Gas6 expression. Both Gas6 mRNA and protein expression down-regulated by P_i were restored by the addition of testosterone. Moreover, flutamide abrogated the increase in Gas6 expression by testosterone in HASMC (Fig. 3, *C* and *D*).

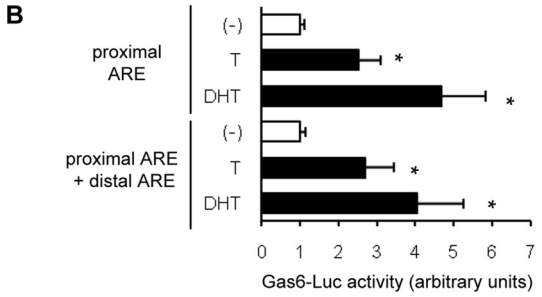
The preventive effect of Gas6 on P_i-induced apoptosis and calcification is mediated by the phosphatidylinositol 3-OH kinase/Akt pathway, a well known anti-apoptotic signaling pathway, through Bcl2 family proteins (17). We found that testosterone restored the Akt phosphorylation down-regulated by P_i, and this increase in phosphorylation was blocked by flutamide (supplemental Fig. 1*A*). Furthermore, SH-5, an Akt inhibitor, abolished the effect of androgens on HASMC calcification (supplemental Fig. 1*B*).

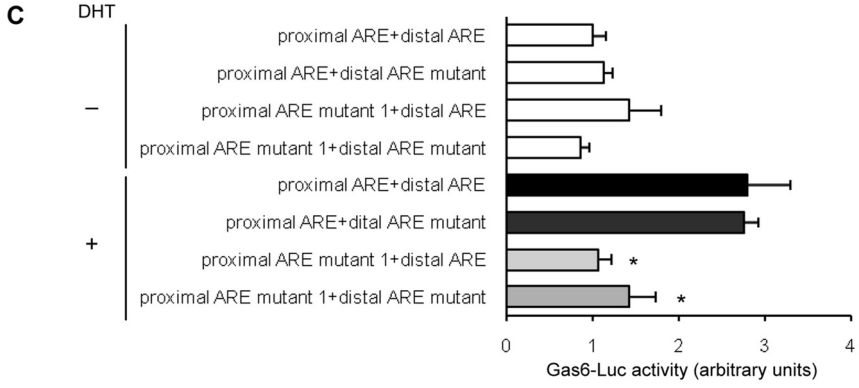
To determine whether Gas6 is required for androgen-mediated effects, we blocked the action of Gas6 using siRNA (Fig. 3*E*) and examined the effect of androgens on P_i-induced calcification. As shown in Fig. 3*F*, knockdown of the Gas6 gene significantly reversed the inhibitory effect of androgens on P_i-induced calcification.

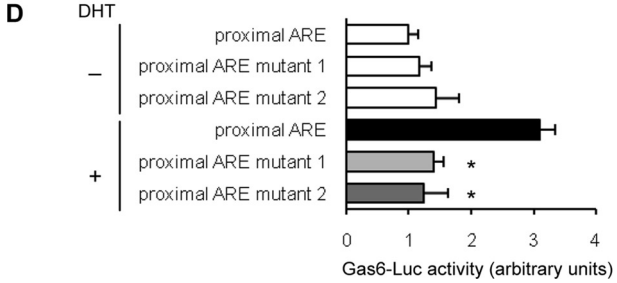
The Proximal ARE in Gas6 Promoter Is Essential for Androgen-stimulated Gas6 Transcriptional Activation—To investigate the molecular mechanism involved in up-regulation of Gas6 expression by androgens, we explored the existence of ARE sites in the promoter region of the Gas6 gene (−1827 to +99 bp). We found that the Gas6 promoter contained two consensus ARE sites. One ARE (−535 to −549 bp) was located close to the transcription start site, whereas the other was located at −1733 to −1748 bp (Fig. 4*A*). To examine whether AREs in Gas6 were functional, we made two constructs; one contained only the proximal ARE site of the Gas6 promoter, and the other contained both the proximal and distal ARE sites. With transient transfection, androgens significantly stimulated Gas6 promoter activity of the proximal ARE, whereas an additional increase in Gas6 promoter activity was not observed by transfection of the construct containing both the proximal ARE and the distal ARE (Fig. 4*B*). Then we performed site-directed mutagenesis to confirm whether the proximal ARE is critical. The distal and proximal ARE sites were mutated, as shown in Fig. 4*A*. Mutation of the proximal ARE completely abrogated DHT-stimulated Gas6 transcription activity. However, we did not observe a reduction in Gas6 transcription activity with the distal ARE mutation (Fig. 4*C*). To further verify the importance of the proximal ARE sequence in androgen-dependent activation of Gas6, we examined two mutants of the proximal ARE. As expected, both of the mutants abrogated DHT-stimulated Gas6 promoter activity, whereas they had no effect in the absence of DHT (Fig. 4*D*). Taking these results together, we identified two ARE sites in the Gas6 promoter and found that the proximal ARE is essential for androgen-induced activation of the Gas6 promoter.

Androgen-dependent Gas6 Promoter Activity Is Mediated by Binding of the AR to the ARE—To examine the role of the AR in androgen-dependent Gas6 promoter activation, we used flutamide and AR siRNA to block the function of the AR. First, we found that flutamide completely eliminated DHT-induced activation of the Gas6 promoter (Fig. 5*A*). However, P_i did not affect Gas6 promoter activity. Next, AR siRNA clearly down-regulated AR protein expression, as shown in Fig. 5*B*. By transient transfection of AR siRNA, Gas6 promoter activity was significantly inhibited in the









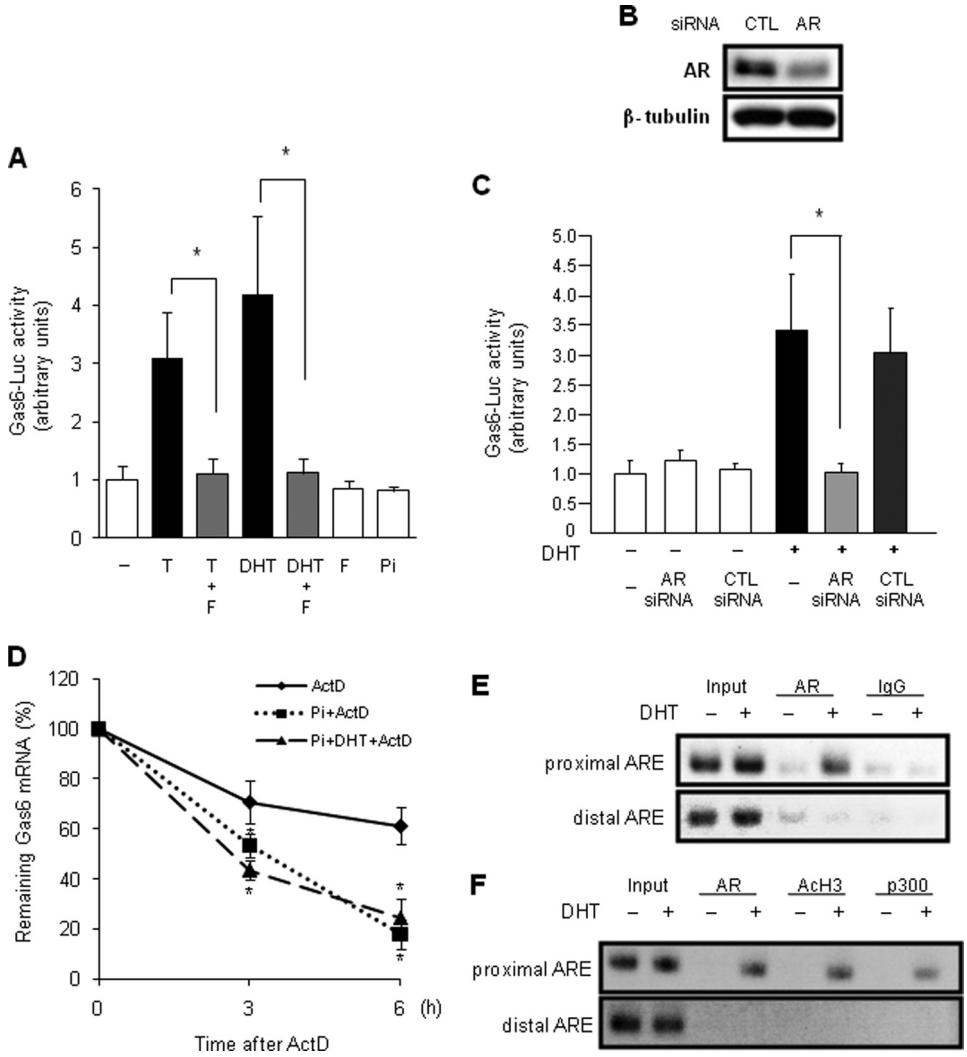


FIGURE 5. **Interaction of the AR with the proximal ARE is essential for transactivation of Gas6 gene by androgen.** *A*, HASMC were transfected with the Gas6-luc construct containing the proximal ARE. Twenty-four hours after transfection, testosterone (*T*, 100 nM), DHT (100 nM), P_i (*Pi*, 2.6 mM), or flutamide (*F*, 10 μM) was added, and the cells were incubated for an additional 24 h. *, $p < 0.05$ by Fisher's test. *B*, HASMC were transfected with AR or control (*CTL*) siRNA (100 nM). The AR protein was efficiently decreased by AR siRNA at 48 h after transfection. *C*, HASMC were transfected with 0.8 μg of Gas6 proximal ARE together with AR siRNA or nonspecific (*CTL*) siRNA (100 nM). Twenty-four hours later, DHT (100 nM) or vehicle was added. After a further 24 h, luciferase activity was assayed. *D*, serum-starved HASMC were incubated with Act D (5 μg/ml) in the presence of 2.6 mM P_i after 12 h of DHT (100 nM) treatment. The remaining Gas6 mRNA was determined at 0, 3, and 6 h after Act D treatment by real-time PCR analysis. Values of Gas6 mRNA with P_i (*dotted line with squares*), with P_i and DHT (*dashed line with triangles*), or without P_i (*solid line*) in the presence of Act D were normalized to that of β-actin mRNA at each time point. Gas6 mRNA level at time 0 was expressed as a percentage of the maximum value. The results are the average of three separate experiments. *, $p < 0.05$ *versus* Act D by Fisher's test. *E*, chromatin extracts were obtained from HASMC after treatment with or without 100 nM DHT for 12 h, and the ChIP assay was performed using an antibody against AR or control IgG. DNA fragments were extracted from immunoprecipitates. The Gas6 promoter region containing proximal ARE was amplified, but distal ARE was not. *F*, a ChIP assay was performed using an antibody against AR, acetylhistone H3 (*AcH3*), or p300 with chromatin extracts with or without treatment with 100 nM DHT for 24 h. Relative promoter activities are expressed as the mean ± S.E. of quadruplicate samples. Similar results were obtained from four independent experiments. *, $p < 0.05$ by Fisher's test.

presence of DHT (Fig. 5*C*). These findings suggest that Gas6 transactivation by androgens was dependent on the AR.

Because P_i did not affect Gas6 transcriptional activity, we further explored the effect of P_i on Gas6 regulation at the post-transcriptional level. The stability of Gas6 mRNA was examined in the presence or absence of Act D. We found that Gas6 mRNA was significantly more degraded in the presence of P_i than in its absence after Act D treatment (Fig. 5*D*). DHT did not have an effect on mRNA degradation (Fig. 5*D*). These findings suggest that P_i down-regulated Gas6 expression by increasing the mRNA degradation rate and not by decreasing transcriptional activity.

To confirm a direct association of the AR with the proximal ARE in the Gas6 gene, we performed a ChIP assay in HASMC. After 12 h of DHT treatment, a polyclonal antibody against the AR could efficiently precipitate the androgen-responsive region of Gas6, showing that the AR directly binds to the Gas6 gene promoter region containing the proximal ARE site in HASMC (Fig. 5*E*). We did not observe binding of the AR to the distal ARE site in the Gas6 gene (Fig. 5*E*). Furthermore, we attempted a characterization of the promoter interactions with an AR-containing transcriptional complex. Histone acetyltransferase, such as p300, is a well established coactivator of the AR, and acetylation of histone H3 is an important determinant of AR action, possibly mediated by p300 (19). We performed a ChIP assay with antibodies against acetylhistone H3 and p300. When the AR binds to the proximal ARE site of the Gas6 gene, acetylhistone H3 and p300 also bind to this site as coactivators (Fig. 5*E*). We did not

FIGURE 4. **Androgens stimulate Gas6 promoter activity in HASMC.** *A*, shown is a schematic representation of the sequence for ARE sites in wild-type human Gas6 promoter and mutant construct. Site-directed mutagenesis was used to alter the ARE sites within the Gas6 construct. The sequences of the consensus ARE site, Gas6 ARE sites, and the mutated ARE sites with altered bases *underlined* are shown. *B*, 24 h after transfection of 0.8 μg of Gas6-luc construct containing only the proximal ARE or the construct containing both the proximal and distal AREs, androgens (testosterone (*T*) and DHT, 100 nM) were added, and the cells were incubated for an additional 24 h. *, $p < 0.05$ *versus* androgens (−) by Fisher's test. *C*, HASMC were treated with DHT (100 nM) or vehicle for 24 h after transfection of the Gas6-luc constructs containing both proximal and distal AREs or mutants. *, $p < 0.05$ *versus* DHT (+) wild-type Gas6 by Fisher's test. *D*, HASMC were transfected with wild-type or two proximal ARE mutants. Twenty-four hours after transfection, DHT (100 nM) was added for an additional 24 h. Luciferase activity was normalized to that of the DHT-free wild-type Gas6 construct. *, $p < 0.05$ *versus* DHT(+) wild-type Gas6 by Fisher's test. Relative promoter activities are expressed as the mean ± S.E. of quadruplicate samples. Similar results were obtained from five independent experiments.

observe any binding of the AR, acetylhistone H3, or p300 to the distal ARE site in the Gas6 gene (Fig. 5*F*).

DISCUSSION

The effect of testosterone replacement therapy on atherosclerosis is controversial (21–25), although testosterone deficiency is known to be associated with cardiovascular disease in men (26–30). We and others have shown that a low testosterone level is associated with markers of atherosclerosis such as impaired endothelial vasomotor function (27), increased carotid intima-media thickness (28), and aortic calcification (9). Recently, testosterone has also been reported to inhibit VSMC proliferation and neointima formation (7), suggesting a direct action of testosterone on the vasculature. In this *in vitro* study we examined the effect of androgens on P_i-induced VSMC calcification and found that androgens at physiological concentrations exhibited inhibitory effects on VSMC calcification. In contrast to the present study, it has been reported that androgens induced vascular calcification in apolipoprotein E knockout mice (31). This discrepancy may derive from the complex *in vivo* effects of testosterone. Further work is required to define the role of androgens in vascular calcification.

Androgens act mainly through transcriptional control of target genes mediated by the nuclear AR (11, 32). In the present study we found that the AR was expressed predominantly in the nucleus of VSMC and had transcriptional activity. Recently, it was demonstrated that the AR-dependent action of androgens protects against angiotensin II-induced vascular remodeling (33). Consistent with this, our results showed that the inhibitory effect of androgens on VSMC calcification was mediated by the AR and not by estrogen receptor.

Recently, we demonstrated that apoptosis plays a central role in the process of P_i-induced VSMC calcification through down-regulation of the Gas6-mediated survival pathway (16, 17). In the present study we found that androgens prevented VSMC apoptosis and restored Gas6 expression and Akt survival signaling. These inhibitory effects of androgens on apoptosis and calcification were eliminated by flutamide and Gas6 siRNA. Our findings indicate that AR-dependent restoration of Gas6 by androgens contributes to the inhibition of apoptosis and VSMC calcification.

Although the involvement of other molecules such as protein kinase Cδ (7) and endothelial nitric-oxide synthase (33) in the vasoprotective actions of androgens is unclear, our data showed that Gas6 plays a pivotal role in the inhibitory effect of androgen on P_i-induced calcification. Several genes containing AREs and having AR-mediated actions have been identified (34, 35). However, little is known about transcriptional regulation and the target genes of the actions of the AR in the vascular system. In this study we identified two AREs in the promoter region of the Gas6 gene and characterized specific direct binding of the AR to the proximal ARE, in contrast to the nonfunctional distal ARE. Interestingly, Mo *et al.* (36) identified that an estrogen response (ER) element spanning −72 to −89 bp from the translation start site in Gas6 and ERα is recruited by estrogen-mediated stimulation of Gas6 gene expression in mouse mammary epithelial cells. In the human Gas6 promoter domain, we also found the existence of an estrogen response element at −243 to −251 bp. In clinical studies, a low serum estradiol level in women was correlated with increased arterial calcification (37), and estrogen replacement could reduce coronary calcification (38, 39). However, in experimental studies, estradiol treatment showed variable effects on vascular calcification with either inhibition (40, 41) or stimulation of calcification (42). Further studies are needed to elucidate the actions of estrogens in vascular calcification.

In summary, this study showed that Gas6 is a novel target that is directly and transcriptionally regulated by the AR, and direct interaction of the AR and Gas6 mediates the inhibitory effects of androgens on vascular calcification. This study provides a new mechanistic insight into the vascular protective action of androgens.

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