Androgen Receptor-Dependent Activation of Endothelial Nitric Oxide Synthase in Vascular Endothelial Cells: Role of Phosphatidylinositol 3-Kinase/Akt Pathway

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The mechanisms of testosterone-induced vasodilatation are not fully understood. This study investigated the effect of testosterone on nitric oxide (NO) synthesis and its molecular mechanism using human aortic endothelial cells (HAEC). Testosterone at physiological concentrations (1–100 nM) induced a rapid (15–30 min) increase in NO production, which was associated with phosphorylation and activation of endothelial NO synthase (eNOS). Then, the involvement of the androgen receptor (AR), which is abundantly expressed in HAEC, was examined. The effect of testosterone on eNOS activation and NO production were abolished by pretreatment with an AR antagonist nilutamide and by transfection with AR small interference RNA. In contrast, testosterone-induced eNOS phosphorylation was unchanged by pretreatment with an aromatase inhibitor or by transfection with ERα small interference RNA. 5α-Dihydrotestosterone, a nonaromatizable androgen, also stimulated eNOS phosphorylation. Next, the signaling cascade that leads to eNOS phosphorylation was explored. Testosterone stimulated rapid phosphorylation of Akt in a time- and dose-dependent manner, with maximal response at 15–60 min. The rapid phosphorylation of eNOS or NO production induced by testosterone was inhibited by Akt inhibitor SH-5 or by phosphatidylinositol (PI) 3-kinase inhibitor wortmannin. Co-immunoprecipitation assays revealed a testosterone-dependent interaction between AR and the p85α subunit of PI3-kinase. In conclusion, testosterone rapidly induces NO production via AR-dependent activation of eNOS in HAEC. Activation of PI3-kinase/Akt signaling and the direct interaction of AR with p85α are involved, at least in part, in eNOS phosphorylation. (***Endocrinology*** **151: 1822–1828, 2010)**

Steroid hormones play various roles in vascular functions through the specific receptor (1). Although the effects of androgens on the cardiovascular system have been controversial (2), recent epidemiological studies have shown that low testosterone level is associated with incident cardiovascular disease (3, 4) and impaired endothelial vasomotor function (5) in men. Several studies have also shown that short-term administration of testosterone to men with coronary artery disease reduces myocardial ischemia (6–8) and improves endothelial vasomotor function (9, 10). These findings suggest beneficial effects of testosterone on the cardiovascular systems and endothelium in men. Testosterone causes acute vasorelaxation *in vitro* and *in vivo* in animals and in humans (11–14), but the precise mechanisms of testosterone-induced vasorelaxation are still unknown. Some suggest the role of nitric oxide (NO) (12, 15), but others have denied the involvement of NO (16, 17).

Androgen receptor (AR) is a member of the nuclear receptor superfamily, which exerts its effects by modifying

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Abbreviations: AR, Androgen receptor; DAF-2DA, 4,5-diaminofluorescin diacetate; DHT, 5α-dihydrotestosterone; eNOS, endothelial NO synthase; ERα, estrogen receptor-α; HAEC, human aortic endothelial cells; L-NAME, nitro-L-arginine methyl ester; NOx, nitrite/nitrate; NT, nontargeting; PI, phosphatidylinositol; siRNA, small interference RNA.

gene expression (18). The signal transduction pathways activated by AR has not been defined well in the cardiovascular system. We have previously reported that AR is involved in endothelial NO synthase (eNOS) activation induced by ginsenoside-Rb1, a steroid hormone-like herb, through phosphatidylinositol (PI) 3-kinase/Akt signaling (19). In the present study, we investigated the effect of testosterone on eNOS activation and NO production and further explored the role of AR and its signaling pathway in human aortic endothelial cells (HAEC).

Materials and Methods

Cell culture

HAEC (derived from a 50-yr-old man, tissue acquisition no. 14506, lot no. 6F3753; Cambrex BioScience, Inc., Walkersville, MD) were maintained in EBM-2 (Clonetics, Walkersville, MD) medium supplemented with 10% fetal bovine serum, a growth factor cocktail [0.1% human epidermal growth factor, 0.04% hydrocortisone, 0.4% human fibroblast growth factor-B, 0.1% vascular endothelial growth factor, 0.1% R^3-IGF-1 [an 83-amino acid analog of IGF-1 comprising the complete human IGF-1 sequence with the substitution of an Arg (R) for the Glu (E) at position three, hence R^3 and a 13-amino acid extension peptide at the N terminus], 0.1% ascorbic acid, 0.1% (Gentamicin, Amphotericin B)-1000, 0.1% heparin], and penicillin (100 U/ml)/streptomycin (100 μg/ml). For the experiments, HAEC at the fifth to seventh passage were seeded in collagen-coated two-chamber slides [for 4,5-diaminofluorescin diacetate (DAF-2DA) experiment], six-well plates [for nitrite/nitrate (NOx) determination], or 6-cm dishes (for immunoblotting and other experiments), respectively, at a concentration of 10^4 cells/cm^2 and grown to subconfluence. Then the cells were incubated in phenol red- and growth factor cocktail-free EBM-2 medium supplemented with 1% dextran-coated charcoal-stripped fetal bovine serum (condition medium) for 6 h to diminish steroids and to obtain growth arrest. In the inhibition experiments, the cells were pretreated with inhibitors for 60 min before the stimuli. Dimethylsulfoxide (0.01–0.02%) was used as a vehicle and a solvent for reagents used in this study.

Detection of NO

NO production was examined using NO-sensitive fluorescent dye DAF-2DA (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Briefly, cells were seeded in collagen-coated two-chamber slides in culture medium (2 ml) until subconfluence. Growth-

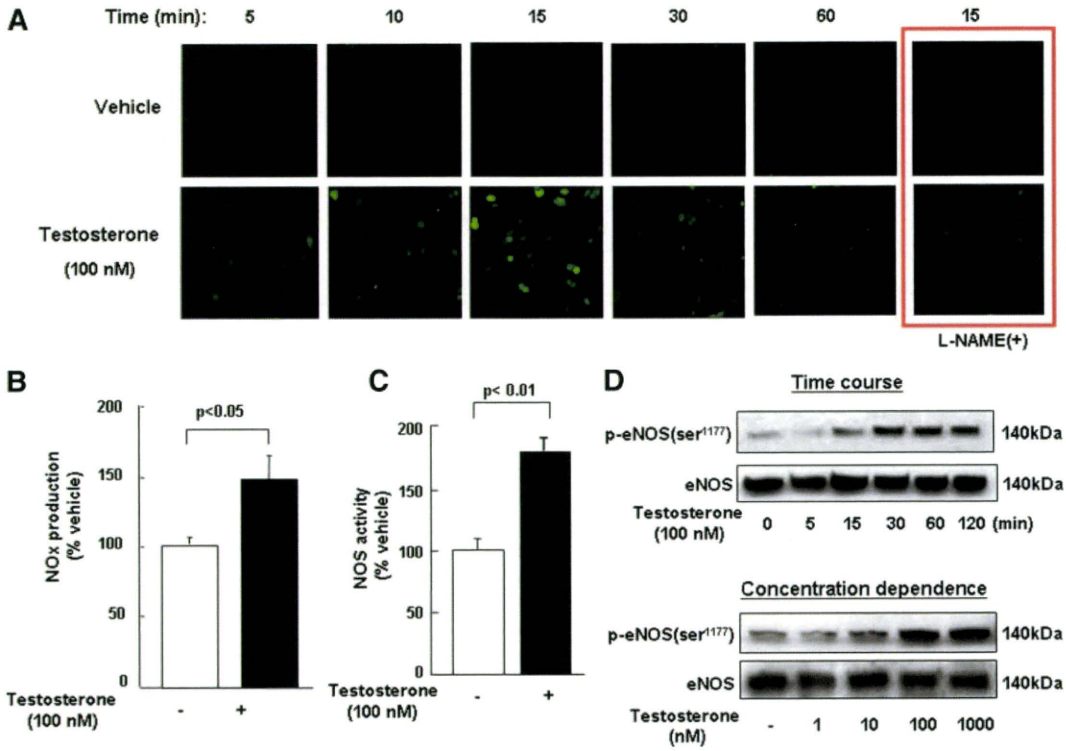

FIG. 1. Testosterone rapidly stimulates NO production and NOS activation in HAEC. A, Growth-arrested HAEC were loaded with DAF-2DA before treatment with or without testosterone for the indicated times. After testosterone treatment, cells were fixed in 2% paraformaldehyde and viewed using a fluorescence microscope. Emission of green light is indicative of NO production. In some groups of cells, L-NAME (0.5 mM) was added 30 min before loading cells with DAF-2DA. B, NOx concentration in the supernatants was measured as stable metabolites of NO at 2 h after stimulation with testosterone. Data were converted to percentage of vehicle and expressed as mean ± SEM of three independent experiments using different cell preparations (n = 4). C, After testosterone or vehicle was added to the medium for 30 min, cells were homogenized, and the activity of NOS was measured by the ability of NOS to convert [^{3}H]L-arginine to [^{3}H]L-citrulline as described in *Materials and Methods*. Data were converted to percentage of vehicle and expressed as mean ± SEM of three independent experiments using different cell preparations (n = 3). D, Growth-arrested HAEC were incubated with testosterone or vehicle for the indicated times or with the indicated concentrations of testosterone for 30 min. Phosphorylation of eNOS at Ser1177 (p-eNOS), and the total eNOS levels in cell lysates were analyzed by immunoblotting. Representative blots are shown, and the results were confirmed by at least three independent experiments. A representative result of at least three independent experiments are shown in A and D.

arrested cells were loaded with DAF-2DA (5 μM for 30 min at 37 C) and then rinsed three times with Hanks' balanced salt solution (Hanks' buffer; Sigma-Aldrich, St. Louis, MO), kept in the dark, and maintained at 37 C in condition medium (2 ml). After 90 min, cells were treated with testosterone or other stimuli. For inhibition experiments, the inhibitors were added 60 min before stimuli. Green fluorescence intensity was visualized with a laser-scanning confocal microscopy system [Bio-Rad (Hercules, CA) Laser Sharp2000] connected to a CCD camera and a computer system. Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production as a result of the reaction of DAF-2DA with NO. Accumulation of NO from HAEC in the culture medium was measured as the levels of NOx, oxidized products of NO, using a fluorometric 2,3-diaminonaphthalene kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). Briefly, cells were seeded in collagen-coated six-well plates in cultural medium (2 ml) until subconfluence. Growth-arrested cells were stimulated with testosterone for 2 h in the condition medium (1 ml/well). Cell- and debris-free supernatants were applied for the measurement to total NOx concentrations. NOx concentrations were calculated according to the manufacturer's protocol.

NOS activity assay

NOS activity was quantified by measurement of the conversion of L-arginine to L-citrulline using an NOS assay kit (Calbiochem, EDM Biosciences, Inc., La Jolla, CA). Briefly, cells were seeded in collagen-coated 6-cm dishes and grown until subconfluent and growth arrested. At 30 min after stimulation with testosterone, cells were harvested and lysed. The concentration of protein in cell lysate was adjusted to 10 μg/μl. According to the manufacturer's protocol, total cell lysate and reaction mixture were incubated with 1 μCi/μl L-[2,3,4,5-^{3}H]arginine (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 60 min at 30 C, and then, converted citrulline was separated from the mixture by passing the mixture through a column of equilibrated cups. The eluate was sampled in scintillation liquid. Extracts incubated with an NOS inhibitor, nitro-L-arginine methyl ester (L-NAME, 1 mM), served as the blank. Converted NOS activity was calculated by subtracting the value of the blank from that of the sample.

Small interference RNA (siRNA) transfection

siRNA duplexes (Santa Cruz Biotechnology, Santa Cruz, CA) against AR (accession no. sc-29204) and estrogen receptor-α (ERα) (accession no. sc-29305) were used for directed knockdown of protein expression. Nontargeting (NT) scrambled siRNA (Santa Cruz; accession no. sc-37007) was used as a control siRNA. HAEC were seeded in collagen-coated 6-cm dishes and grown in culture medium without antibiotics. At 50–60% confluence, cells were transfected with 5 nM AR siRNA, 10 nM

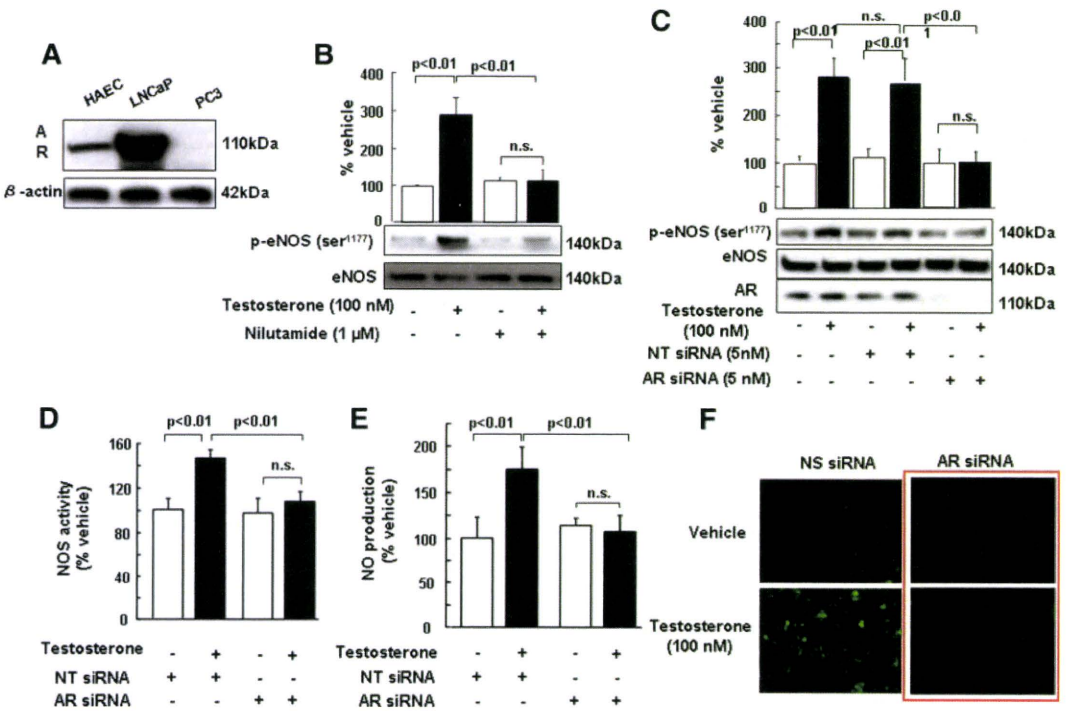

FIG. 2. AR is involved in testosterone-induced eNOS phosphorylation. A, AR expression was examined in HAEC, AR-positive LNCaP cells, and AR-negative PC3 cells using immunoblotting (*upper panel*). The membrane was stripped and re-immunoblotted with anti-β-actin antibody for monitoring equal amounts of protein from each sample (*lower panel*). B, Cells were treated with testosterone or vehicle for 30 min in the presence or absence of nilutamide. Phosphorylation of eNOS at Ser1177 (p-eNOS), and protein levels of eNOS were analyzed using immunoblotting. C–F, Growth-arrested HAEC were transfected with 5 nM AR siRNA or 5 nM nontargeting scrambled (NT) siRNA for 24 h as described in *Materials and Methods*. Cells were treated with testosterone or vehicle for 30 min. C, Phosphorylated eNOS at Ser1177 (p-eNOS) and total eNOS and AR were analyzed using immunoblotting. D, NOS activity was measured by the ability of NOS to convert [^{3}H]L-arginine to [^{3}H]L-citrulline as described. E, NOx concentration in the supernatants was measured as described in *Materials and Methods*. F, After transfection with siRNA, growth-arrested cells were loaded with DAF-2DA and treated with or without testosterone for 15 min. NO production was examined as described in *Materials and Methods*. A representative result from three independent experiments is shown in A–C and F. Data represent mean ± SEM of the p-eNOS/eNOS ratio of quantified densities from three independent experiments; representative blots are shown in B and C. Data were converted to fold over vehicle and expressed as mean ± SEM of three independent experiments using different cell preparations (n = 3) in D and E. n.s., Not significant.

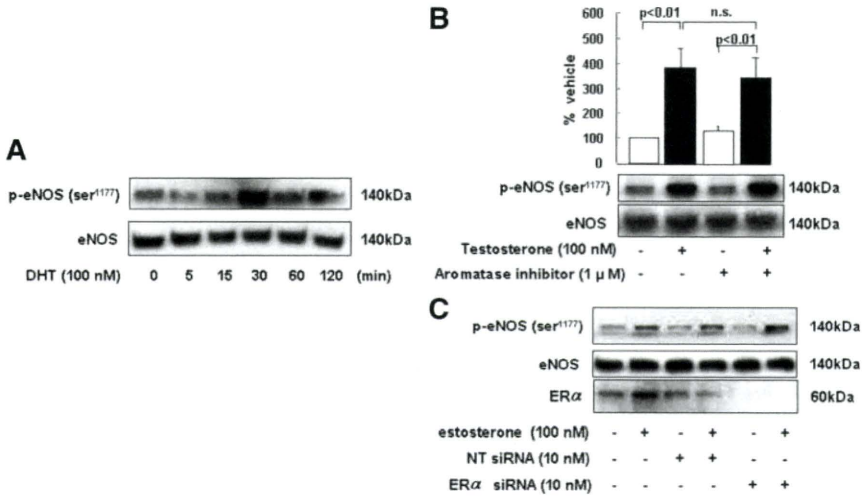

FIG. 3. Estradiol/ER pathway is not involved in testosterone-induced eNOS phosphorylation. A, Growth-arrested HAEC were treated with DHT or vehicle for the indicated times. B, Growth-arrested cells were treated with testosterone or vehicle for 30 min in the presence or absence of aromatase inhibitor. Data represent mean ± SEM of the phosphorylated eNOS (p-eNOS)/eNOS ratio of quantified densities from three independent experiments. A representative blot is shown. n.s., Not significant. C, Cells were treated with testosterone or vehicle for 30 min after transfection of siRNA against ERα or nontargeting scrambled (NT) siRNA (10 nM). A–C, Phosphorylation of eNOS at Ser1177 (p-eNOS), total eNOS, and ERα in cell lysates were analyzed using immunoblotting. A representative result from three independent experiments is shown.

ERα siRNA, or NT siRNA using HiPerFect transfection reagent (QIAGEN, Valencia, CA) in 1 ml transfection medium (Santa Cruz). Two hours later, 3 ml culture medium was added, and incubation was performed for another 22 h. Cells were washed with Hanks' buffer and used for the experiments.

Immunoprecipitation and immunoblotting

Immunoprecipitation assays were performed according to the standard protocol. Briefly, treated cells were washed twice in ice-cold Hanks' buffer and lysed with RIPA lysis buffer (Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.004% sodium azide, protease inhibitor cocktail, and sodium orthovanadate). The volume of all samples was brought up to 1 ml by the addition of lysis buffer. Equal amounts of total cell lysates (800 μg protein per sample) were precleared with protein A/G agarose beads (Santa Cruz) at 4 C for 30 min and then incubated with anti-p85α antibody (B-9, 1 μg/mg cellular protein; Santa Cruz) at 4 C overnight with end-over-end rotation. A cell lysate with antimouse IgG antibody served as a negative control. After this period, protein A/G agarose beads were added and incubated for another 2–4 h at 4 C. After removal of the lysates by centrifugation, the immunoprecipitates were washed four times with PBS containing 1% Nonidet P-40 and resuspended in 2% electrophoresis sample buffer (Santa Cruz), separated on precast SDS-PAGE, and transferred to polyvinyl difluoride membranes.

Antibodies against AR (N-20; Santa Cruz), ERα (Ab-10; Thermo Fisher Scientific, Fremont, CA), phospho-eNOS (Ser1177), and eNOS/NOS type III (BD Transduction Laboratories, Lexington, KY), phospho-Akt and Akt (Cell Signaling Technology, Beverly, MA) were used for immunoblotting as described previously (19).

Data analysis

Values are expressed as mean ± SEM in the text and figures. Statistical comparisons were performed using ANOVA with *post hoc* Fisher's protected least significant difference test. Differences with a value of $P < 0.05$ were considered statistically significant.

Results

Testosterone stimulates rapid NO production and eNOS activation in HAEC

After the addition of testosterone (Sigma-Aldrich), a significant increase in green fluorescence was observed at 5 min. Maximal stimulation of NO production was observed at 15–30 min, but this response was abolished by pretreatment of cells with an NOS inhibitor, L-NAME (Fig. 1A). The concentration of NOx, stable metabolites of NO, in the culture medium was also increased up to approximately 1.5-fold by testosterone (100 nM) treatment for 2 h (Fig. 1B). Testosterone increased NOS activity, as measured by determination of L-citrulline converted from L-arginine, after 30 min of treatment (Fig. 1C). Also, testosterone induced eNOS phosphorylation (Ser1177) in a time- and dose-dependent manner, with no influence on eNOS protein levels (Fig. 1D).

AR mediates testosterone-induced eNOS activation

We investigated the role of AR in the effect of testosterone on eNOS activation. First, we confirmed the endogenous expression of AR in HAEC by immunoblotting in comparison with an AR-positive prostate cancer cell line, LNCaP, and an AR-negative cell line, PC3 (Fig. 2A). Pretreatment with nilutamide, an AR antagonist, abolished the testosterone-induced rapid phosphorylation of eNOS (Fig. 2B). Next, we applied siRNA for loss-of-function analysis of AR. Associated with the efficient knockdown of AR expression, testosterone-induced eNOS phosphorylation (Fig. 2C), NOS activation (Fig. 2D), and NO production (Fig. 2, E and 2F) were abolished by transfection with AR siRNA but were not by control NT siRNA. We then examined the effect of nonaromatizable 5α-dihydrotestosterone (DHT, 100 nM; Sigma-Aldrich) on eNOS phosphorylation. DHT also induced eNOS phosphorylation (Fig. 3A). In contrast, conversion to estradiol or ERα does not seem to play a role, because neither the aromatase inhibitor (1 μM) nor transfection of ERα siRNA affected testosterone-induced eNOS phosphorylation (Fig. 3, B and C).

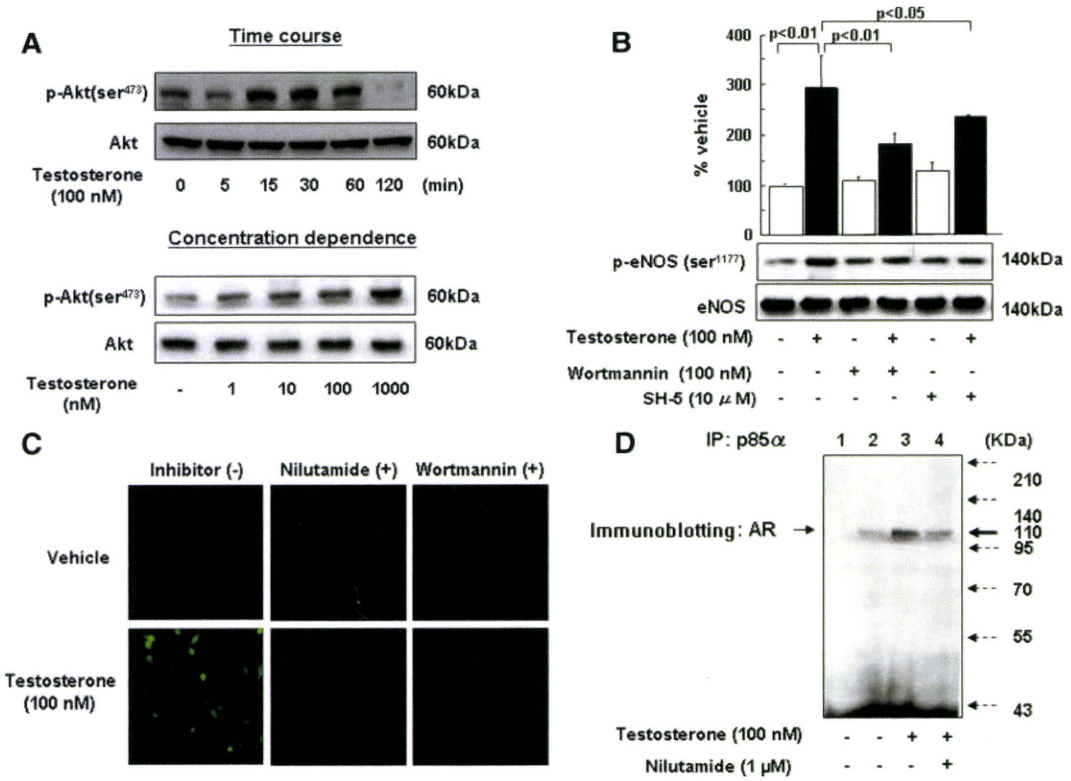

FIG. 4. PI3-kinase/Akt pathway is involved in testosterone-induced eNOS phosphorylation and NO production. A, Growth-arrested HAEC were treated with vehicle or testosterone for the indicated times or with the indicated concentrations of testosterone for 30 min. Phosphorylation of Akt at Ser473 (p-Akt) and total Akt level in cell lysates were analyzed by immunoblotting. B, Wortmannin or SH-5 was added 60 min before the cells were treated with testosterone for 30 min. Phosphorylation of eNOS at Ser-1177 (p-eNOS), and total eNOS levels in cell lysates were analyzed. Data represent mean ± SEM of the p-eNOS/eNOS ratio of quantified densities from three independent experiments. A representative blot is shown. C, Growth-arrested cells were loaded with DAF-2DA and treated with or without testosterone for 15 min in the presence or absence of wortmannin or nilutamide. D, Growth-arrested HAEC were treated with testosterone or vehicle for 30 min in the presence or absence of nilutamide. Cell extracts were immunoprecipitated with anti-p85α antibody (lanes 2–4) or IgG (lane 1) and separated by SDS-PAGE, and AR was detected with anti-AR antibody. A representative result from three independent experiments is shown in A, C, and D.

PI3-kinase/Akt pathway is involved in eNOS phosphorylation and NO production induced by testosterone

Akt, a serine/threonine kinase, is a key effector of PI3-kinase signaling, directing eNOS phosphorylation (20, 21). Therefore, we examined the role of PI3-kinase/Akt in testosterone-induced eNOS phosphorylation. Testosterone rapidly phosphorylated Akt in a time- and dose-dependent manner, with no influence on Akt protein levels (Fig. 4A). Pretreatment with a PI3-kinase inhibitor wortmannin or an Akt inhibitor SH-5 significantly attenuated testosterone-induced eNOS phosphorylation (Fig. 4B). NO production was also inhibited by wortmannin (Fig. 4C).

PI3-kinase consists of the regulatory subunit p85α and the catalytic subunit p110 (22, 23), and the direct interaction between ERα and p85α initiates eNOS activation (24). Thus, we examined whether AR could interact with p85α, using co-immunoprecipitation assays. As shown in Fig. 4D, AR was associated with p85α in a ligand-dependent and AR antagonist-sensitive manner. These results indicate that testosterone stimulates AR binding to the p85α subunit of PI3-kinase, leading to Akt and eNOS activation.

Discussion

In the present study, we demonstrated that testosterone rapidly induces NO production, associated with the phosphorylation/activation of eNOS. Endothelium-derived NO has been shown to modulate a variety of vascular functions, including vasodilation, inhibition of endothelial cell death, inhibition of platelet aggregation, and attenuation of leukocyte infiltration (25). Thus, NO production by testosterone may account, at least in part, for the vasodilatory (11–14) and antiischemic (6–8) action of testosterone as well as the association of endogenous testosterone with endothelial vasomotor function (5) and protection against cardiovascular disease (3, 4) in men.

In endothelial cells, eNOS is a key enzyme for NO production upon the conversion of the substrate L-arginine to L-citrulline (26), and the phosphorylation of eNOS at

serine-1177 is known to increase enzyme activity (27–29). The increase of NO production by testosterone was abolished by pretreatment of cells with an NOS inhibitor, L-NAME (Fig. 1A), indicating the involvement of eNOS in testosterone-stimulated NO production.

Although most of the biological actions of testosterone may be mediated by AR, some of them may be mediated by ER, after conversion to estradiol (30, 31). Indeed, estrogens have been shown to rapidly activate eNOS and stimulate NO production in an ERα-dependent manner (24, 32). In our study, however, the role of both aromatase and ERα in testosterone-induced eNOS phosphorylation was negligible. In addition, nonaromatizable DHT also elicited a significant eNOS phosphorylation. Moreover, eNOS phosphorylation, NOS activation, and NO production induced by testosterone were reversed by pretreatment with an AR antagonist nilutamide or by transfection of AR siRNA. Taken together, it can be concluded that the effects of testosterone on eNOS phosphorylation and NO production are AR dependent. In our preliminary experiments, the expression of AR protein was not altered up to 4 h after testosterone or DHT treatment, although AR expression appeared to increase 24 h later (data not shown). Accordingly, the level of AR expression may not have influenced the rapid response of eNOS and NO to testosterone.

Multiple signal transduction pathways, including phosphoinositide-3 kinase /Akt kinase converge to regulate eNOS activity by phosphorylation (20, 33). In the present study, we showed that testosterone-induced eNOS phosphorylation is activated by the PI3-kinase/Akt pathway. We also demonstrated that AR interacts with the regulatory subunit p85α of PI3-kinase in endothelial cells. The binding was increased by testosterone and inhibited by an AR antagonist. Although we have not identified the sites of interaction between AR and p85α, Sun *et al.* (34) have demonstrated that the N terminus of AR binds to the C-terminal Src-homology 2 domain (SH2 domain) of p85α in LNCaP cells. Accordingly, AR might bind to p85α in a similar fashion in endothelial cells.

Others have also investigated the mechanisms underlying the vasodilatory action of testosterone (15–18). Their results have been controversial, although many studies suggest the involvement of NO. For instance, Chou *et al.* (12) and Costarella *et al.* (15) reported that in canine coronary arteries and rat thoracic aorta, relaxation in response to testosterone was attenuated by a NOS inhibitor, L-NAME (10, 11), suggesting a role for NO. In contrast, Honda *et al.* (16) and Tep-areenan *et al.* (17) have shown that vasorelaxation to testosterone is endothelium dependent but is not L-NAME sensitive. Yue *et al.* (11) and others (1, 35, 36) demonstrated that vasodilatation induced by pharmacological concentrations of testosterone is independent of the vascular endothelium and AR. The different mechanistic findings underlying the vasodilatory action of testosterone between our study and others (11, 16, 17, 35, 36) are unclear but may be attributable to the experimental conditions such as type of cells, tissue preparations, dose of testosterone, and inhibitors used. In our study, we used primary cultured HAEC; thus, we did not examine how produced NO acts on the arterial wall and vascular smooth muscle cells. Additional studies are required for better understanding of actions of testosterone on the cardiovascular system and the role of AR and its signaling pathways.

In summary, we found that testosterone *per se* stimulates rapid production of NO through AR-dependent activation of eNOS in HAEC. Activation of PI3-kinase/Akt signaling and the direct interaction of AR with p85α are involved, at least in part, in the phosphorylation of eNOS.

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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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[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.

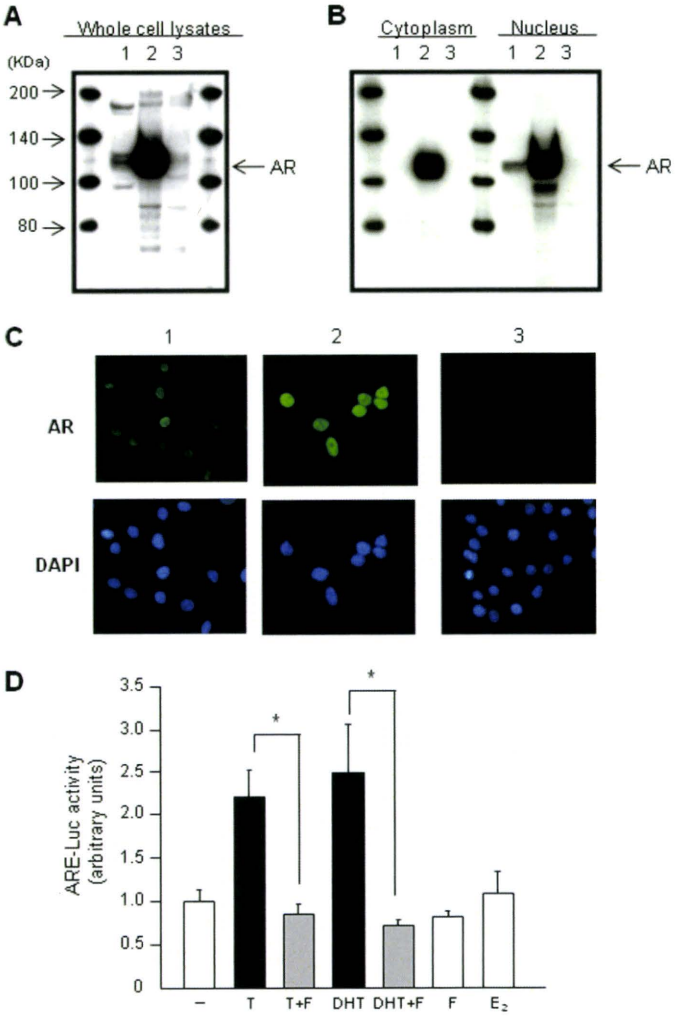


FIGURE 1. **Expression of the functional the AR in HASMC.** Endogenous expression of the AR in HASMC (*lane 1*) was examined in whole cell lysates (*A*) and cytoplasmic and nuclear fractions (*B*) compared with that in human prostate cancer cell lines, LNCaP (AR-positive; *lane 2*) and PC-3 (AR-negative; *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*).

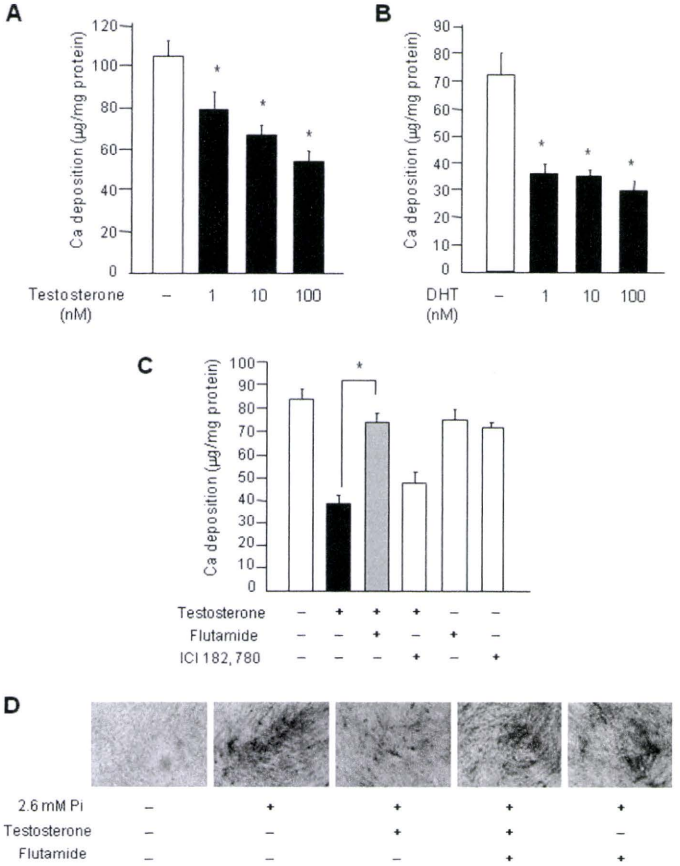


FIGURE 2. **Androgens prevent P_i-induced calcification via the AR.** HASMC were cultured with the indicated concentrations of androgens (testosterone (*A*) and DHT (*B*)) in the presence of 2.6 mM P_i for 6 days. Calcium deposition was measured by the *o*-cresolphthalein complexone method and normalized 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.

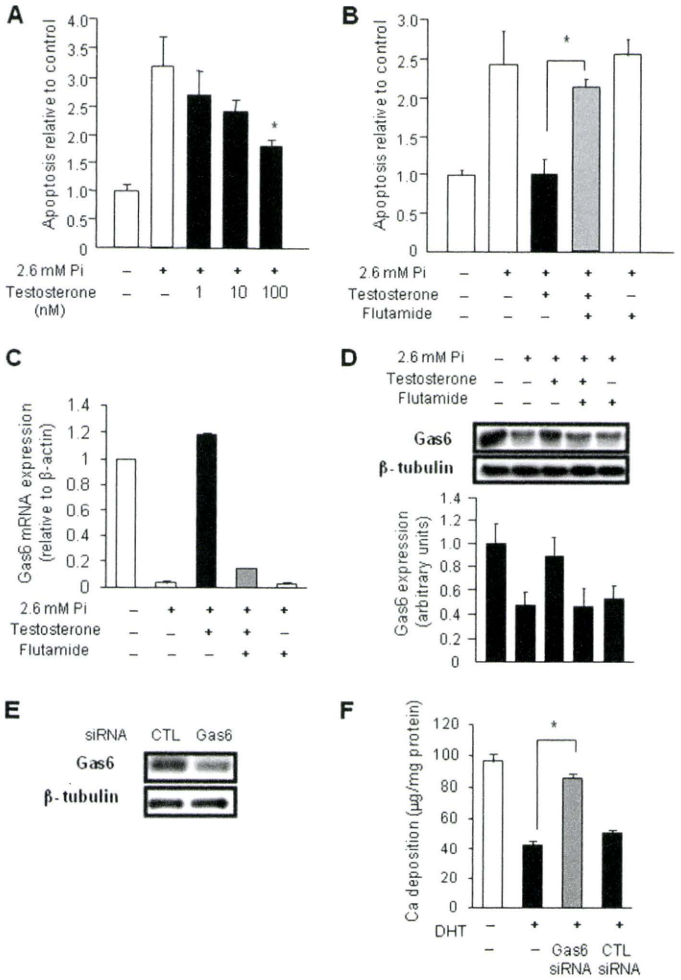


FIGURE 3. **Androgens inhibit P_i-induced apoptosis and restore Gas6-mediated survival pathway.** *A*, HASMC were cultured with the indicated concentrations of testosterone in the presence of 2.6 mM P_i for 6 days. A quantitative index of apoptosis, determined by DNA fragmentation enzyme-linked immunosorbent 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.

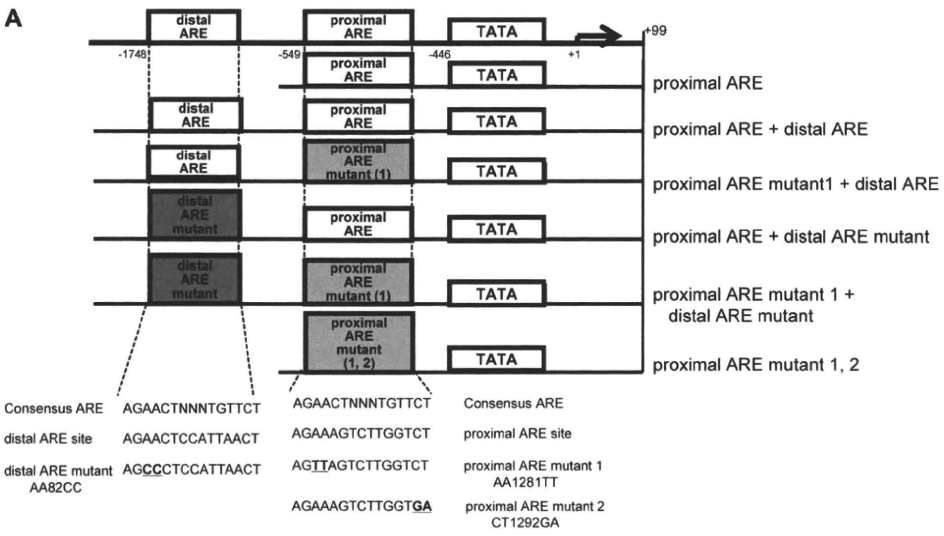
Because apoptosis is a crucial and initiating event in P_i-induced VSMC calcification (16, 17), we examined whether androgens inhibit P_i-induced apoptosis. Furthermore, in our recent study apoptosis induced by P_i has been shown to be associated with inhibition of Gas6 expression and secretion (16, 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



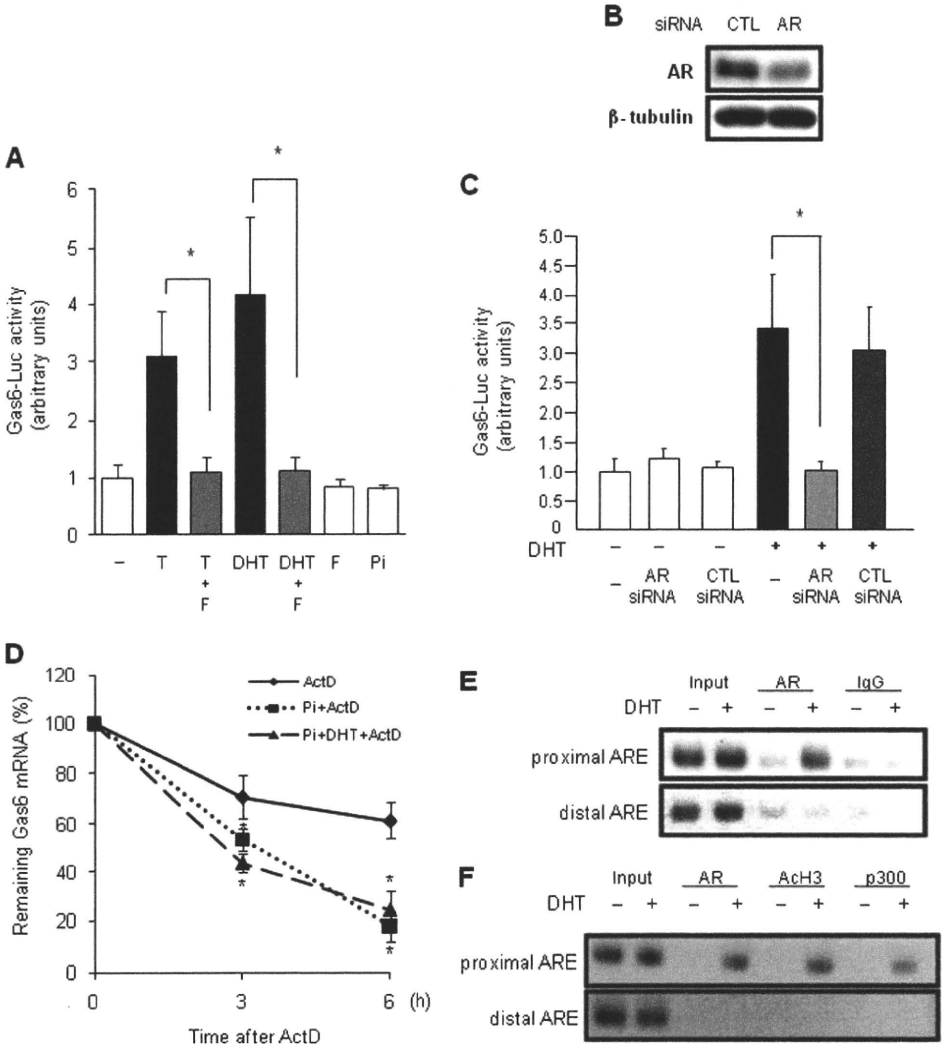


FIGURE 5. **Interaction of the AR with the proximal ARE is essential for transactivation of Gas6 gene by androgen.** *A*, HASMC were transfected with the Gas6-luc construct containing the proximal ARE. Twenty-four hours after transfection, testosterone (*T*, 100 nM), DHT (100 nM), P_i (*Pi*, 2.6 mM), 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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Low testosterone level as a predictor of cardiovascular events in Japanese men with coronary risk factors

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Objective: Recent epidemiological studies have found that testosterone deficiency is associated with higher mortality largely due to cardiovascular (CV) disease in community-dwelling older men. We investigated whether a low plasma testosterone level could predict cardiovascular events in middle-aged Japanese men with coronary risk factors.

Methods: One hundred and seventy-one male outpatients (30–69 years old, mean ± SD = 48 ± 13 years) who had any coronary risk factor (hypertension, diabetes, dyslipidemia, smoking, and obesity) without a previous history of CV disease were followed up. At baseline, the subjects underwent examination of coronary risk factors, measurement of flow-mediated dilation (FMD) of the brachial artery as an indicator of vascular endothelial function and assays of plasma total testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol.

Results: During the mean follow-up period of 77 months, a total of 20 CV events occurred. Kaplan–Meier survival analysis by tertile of plasma hormone levels revealed that the subjects with the lowest testosterone tertile were more likely to develop CV events than those with the highest tertile ($P < 0.01$ by log-rank test). Cox proportional hazards models showed that the subjects with the lowest tertile of plasma testosterone (<14.2 nmol/L) had an approximately 4-fold higher CV event risk compared to those with the higher testosterone tertiles after adjustment for coronary risk factors including medication and FMD (unadjusted hazard ratio, 3.61; 95% CI, 1.47–8.86: multivariate-adjusted hazard ratio, 4.61; 95% CI, 1.02–21.04). Multivariate analysis did not show any significant association of DHEA-S, estradiol or cortisol with CV events.

Conclusions: A low plasma testosterone level is associated with CV events in middle-aged Japanese men, independent of coronary risk factors and endothelial function. This is the first report to show the relationship between endogenous testosterone and CV events in Asian population.

1. Introduction

Plasma testosterone level declines with advancing age in men [1]. Testosterone deficiency is often associated with age-related diseases such as erectile dysfunction, osteoporosis, depressed mood, cognitive impairment and frailty [2,3]. Furthermore, a number of studies suggest that testosterone deficiency is related to cardiovascular (CV) disease and its risk factors in men. Inverse relations between testosterone level and coronary risk factors including obesity [4,5], hypertension [5,6], dyslipidemia [4,5], and diabetes [5,7] have been reported. In addition, we and others have shown that a low testosterone level is associated with markers of atherosclerosis such as impaired endothelial vasomotor function [8], increased carotid intima-media thickness [9] and aortic calcification [4]. Although these data do not indicate a causal relationship between endogenous testosterone and CV disease, recent epidemiological studies have demonstrated that community-dwelling older men with a low testosterone level are more likely to die [10–12], largely due to CV disease [11,12]. However, this issue remains unknown in Asian population.

Based on these backgrounds, we tested the hypothesis that a low testosterone level is an independent risk factor for CV disease even in middle-aged Japanese men with coronary risk factors. For this purpose, we conducted a survey of 171 male patients by using baseline clinical information and by measuring sex hormone levels in stored plasma.

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2. Methods

2.1. Subjects

Male subjects aged 30–69 years at baseline, who were referred to our department to check for CV disease and undergo examination of vasomotor function of the brachial artery in 1996–2000, and had any of the classical coronary risk factors including hypertension, dyslipidemia, diabetes mellitus and current smoking, were eligible. Hypertension, dyslipidemia and diabetes mellitus were defined according to diagnostic criteria [13–15] or if the subject was taking any medication for these diseases. Subjects with a history of CV disease, including stroke, coronary heart disease, congestive heart failure and peripheral arterial disease, were excluded. Malignancy, overt endocrine disease and use of steroid hormones were also excluded, because these conditions may have a significant influence on both plasma sex hormones and clinical course.

Of the 188 eligible subjects whose plasma was stored, written informed consent was obtained from 171 subjects; 1 subject refused and 16 subjects were lost to follow-up. Then, plasma hormone levels were measured and follow-up data were obtained in 171 subjects. The study protocol was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo. Each subject or a family member, if the subject had died, gave written informed consent for enrollment in this study.

2.2. Clinical measurements

Clinical information was collected at baseline when each patient attended our department. Blood sampling and measurement of height, weight, blood pressure and vasomotor function were performed in the morning after a 14-h overnight fast. Blood pressure was measured at least twice using an automated, digital electrosphygmomanometer (Omron Healthcare Co., Ltd., Kyoto, Japan) on the nondominant arm in a sitting position, and the average was used for analysis.

Serum total cholesterol and triglyceride concentrations were measured enzymatically, and serum high-density lipoprotein (HDL) cholesterol concentration was measured by the heparin-$Ca^{2+}Ni^{2+}$ precipitation method. Plasma glucose concentration was assayed by the glucose oxidase method, and hemoglobin A1c level was measured by high-performance liquid chromatography.

Plasma concentrations of total testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol were determined using sensitive radioimmunoassays by a commercial laboratory (SRL, Inc., Tokyo, Japan). Because the plasma used for hormone assays was deep-frozen (−80 °C) for up to 7 years, we checked the change in titers using the stored samples, which had been measured at sampling 5–7 years before. Pearson's correlation coefficient between the two measurements was 0.965 for estradiol ($n = 34$), 0.976 for testosterone ($n = 20$), 0.991 for DHEA-S ($n = 15$) and 0.937 for cortisol ($n = 16$), indicating that there was no significant change in plasma titers in our frozen samples. The intra-assay coefficients of variation for the measurements were less than 5%.

Vasomotor function of the brachial artery was evaluated using an ultrasound machine according to the method described previously [16]. Briefly, endothelium-dependent flow-mediated vasodilation (%FMD) was measured as the maximal percent change in the vessel diameter after reactive hyperemia. Subsequently, endothelium-independent nitroglycerin-induced vasodilation was measured as the maximal percent change in the vessel diameter after sublingual administration of nitroglycerin spray (0.3 mg; Toa Eiyo Co., Tokyo). The same examiner (M.H.) performed the measurements of FMD throughout this study.

2.3. Follow-up

The subjects were followed in 2006–2007 by mail and/or visits to our clinic. Each subject or a family member completed the questionnaire on CV disease and health status. CV events analyzed as the endpoints of this study included stroke, coronary artery disease, sudden cardiac death, and peripheral arterial disease. If CV events were reported on the questionnaire, we attempted to confirm the diagnosis of each event by medical records and/or interview by research doctors who were unaware of the patient's plasma hormone levels. Finally, after thorough examination, 20 cases were determined as CV events. Eighteen cases were ascertained by medical records which included clinical course, physical examination, laboratory tests and imagings. Because medical records were not available on other two cases of self-reported ischemic stroke, they were diagnosed according to the phone interview to each patient.

2.4. Data analysis

Values are expressed as mean ± SD in the text unless otherwise stated. Differences between the groups were analyzed using ANOVA for continuous variables and Chi-squared test for categorical variables. Survival was analyzed using Kaplan–Meier plots and log-rank tests. Hazard ratios (HRs) for CV events were analyzed using Cox proportional hazards regression. A value of $P < 0.05$ was considered statistically significant. Data were analyzed using SPSS (Ver. 17.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Characteristics of subjects according to plasma testosterone level

Table 1 shows the baseline characteristics of the subjects by tertile of plasma testosterone. As reported previously [4–8], subjects with the lowest testosterone tertile tended to be obese, hypertensive, dyslipidemic, diabetic, and to have impaired endothelial vasomotor function compared to those with higher testosterone tertiles. Age and smoking status were not different between the groups.

3.2. CV events and hormones

During the mean follow-up period of 77 ± 46 months (median = 54 months), a total of 20 CV events occurred (Table 2). Eleven cases of coronary artery disease included three of myocardial infarction, three of medically treated angina pectoris, four of percutaneous coronary intervention, and one of coronary artery bypass grafting. All of the five cases of stroke were due to cerebral infarction.

As shown in Fig. 1, Kaplan–Meier survival analysis by tertile of plasma testosterone level revealed that low testosterone was associated with CV events. Cox proportional hazards models showed that the subjects with the lowest tertile of plasma testosterone, but not those with the middle tertile, had significantly increased risk for CV events compared to those with the highest tertile (Table 2). Adjustment for age and body mass index did not attenuate the effect.

Then, HRs for the lowest tertile of plasma testosterone vs. the higher (middle and highest) tertiles were analyzed. The subjects with the lowest tertile (<14.2 nmol/L) showed an unadjusted HR of 3.61 (95% CI, 1.47–8.86), and an adjusted HR of 4.24 (95% CI, 1.67–10.78) for age, body mass index, and current smoking. The HR was 4.61 (95% CI, 1.02–21.04) after adjustment for age, body mass index, current smoking, systolic blood pressure, HDL cholesterol, non-HDL cholesterol, hemoglobin A1c, %FMD,

Table 1
Baseline characteristics of subjects by tertile group of plasma testosterone.

	Tertile 1 <14.2 nmol/L (n=57)	Tertile 2 14.2–19.4 nmol/L (n=57)	Tertile 3 >19.4 nmol/L (n=57)	p for trend
Testosterone (nmol/L)	11.0±3.0	17.0±1.6	24.0±3.0	<0.001
(ng/dL)	(318±86)	(490±45)	(693±86)	
DHEA-S (μmol/L)	4.94±2.68	4.55±2.25	4.83±2.64	0.81
Estradiol (pmol/L)	115±30	116±31	133±30	0.004
Cortisol (nmol/L)	386±138	378±142	361±120	0.67
Age (years)	47±13	45±13	50±14	0.24
Body mass index (kg/m^2)	27.6±5.5	25.6±4.3	24.1±3.6	<0.001
Systolic blood pressure (mmHg)	131±18	125±16	123±12	0.01
Diastolic blood pressure (mmHg)	79±15	74±11	74±9	0.04
Non-HDL cholesterol (mmol/L)	4.19±1.27	3.91±1.06	3.74±1.01	0.10
HDL cholesterol (mmol/L)	1.20±0.36	1.23±0.41	1.44±0.48	0.005
Triglycerides (mmol/L)	2.04±2.12	1.91±1.85	1.46±1.28	0.18
Fasting plasma glucose (mmol/L)	6.00±1.18	5.73±0.92	5.73±1.28	0.34
Hemoglobin A1c (%)	5.9±1.7	5.2±0.8	5.5±1.2	0.03
%FMD	4.2±2.7	5.7±4.2	6.1±3.8	0.01
%NTG	12.8±4.3	14.2±5.4	13.2±5.0	0.30
Hypertension, n (%)	30 (53)	20 (35)	20 (35)	0.09
Dyslipidemia, n (%)	33 (58)	35 (61)	24 (42)	0.09
Diabetes mellitus, n (%)	15 (26)	7 (12)	9 (16)	0.13
Current smoker, n (%)	28 (49)	25 (44)	29 (51)	0.74

DHEA-S, dehydroepiandrosterone-sulfate; HDL, high-density lipoprotein; %FMD, percent flow-mediated dilation of brachial artery; %NTG, percent nitroglycerine-induced dilation of brachial artery.
Values are expressed as mean ± SD. Continuous variables were compared by ANOVA and categorical variables by Chi-squared test.

Table 2
Cardiovascular events by tertile of plasma testosterone.

	Tertile 1 <14.2 nmol/L (n=57)	Tertile 2 14.2–19.4 nmol/L (n=57)	Tertile 3 >19.4 nmol/L (n=57)	Total (n=57)
Number of events				
Stroke	2	3	0	5
Coronary artery disease	7	2	2	11
Sudden cardiac death	2	0	0	2
Peripheral arterial disease	1	0	1	2
Total cardiovascular events	12	5	3	20
HRs (95% CI) for total cardiovascular events				
Unadjusted	4.82 (1.36, 17.12)	1.67 (0.40, 6.99)	1(Ref)	
Adjusted for age	6.36 (1.78, 22.80)	1.82 (0.43, 7.71)	1(Ref)	
Adjusted for age and BMI	7.01 (1.94, 25.34)	1.86 (0.44, 7.86)	1(Ref)	

BMI, body mass index. HRs (Hazard ratios) were analyzed using Cox proportional hazards regression.

medications (antihypertensives, statins, hypoglycemic agents and antiplatelet agents), estradiol and DHEA-S. In addition to testosterone, age (HR per year, 1.12; 95% CI, 1.05–1.20), %FMD (HR per 1% increase, 0.80; 95% CI, 0.64–0.99) and HDL cholesterol (HR per 1 mg/dL, 0.88; 95% CI, 0.81–0.95) were independently associated with CV events, but other variables were not in this final model. Further inclusion of other hormones and nitroglycerin-induced endothelium-independent vasodilation into the model did not influence the statistical results (data not shown).

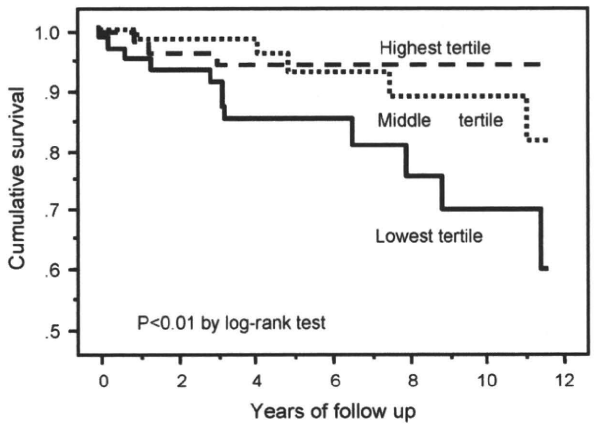

Fig. 1. Survival curves for cardiovascular events by tertile group of plasma concentration of testosterone. Cut-offs of the tertiles for testosterone were 14.2 and 19.4 nmol/L (410 and 560 ng/dL).

Two subjects with the lowest tertile of plasma testosterone suffered CV events within 6 months of follow-up; a case of sudden cardiac death and a case of coronary artery bypass grafting. Accordingly, similar statistical analyses were performed excluding these two cases. The results were essentially unchanged, although the HRs were slightly smaller (unadjusted HR, 3.06; 95% CI, 1.21–7.78: multivariate-adjusted HR, 3.80; 95% CI, 1.06–13.52).

Among other hormones examined, only DHEA-S was associated with increased risk for CV events, but was canceled by adjustment for age (data not shown). Further multivariate analysis did not show any significant association of DHEA-S, estradiol or cortisol with CV events.

4. Discussion

In this follow-up study of middle-aged Japanese men with coronary risk factors, a low plasma testosterone level was associated with CV events. Although the subjects with lower testosterone levels had worse profiles of coronary risk factors [4–7,11,12] and endothelial function [8] at baseline, as reported previously, adjustment for these confounders including age and cardiovascu-

lar medication indicated that low testosterone was an independent risk factor for CV events. In contrast, DHEA-S, estradiol and cortisol levels were not related to CV events.

A number of cross-sectional studies have shown an association between low testosterone level and CV disease [17,18], but have not provided evidence of a causal relationship between them. In recent years, longitudinal follow-up studies have demonstrated that community-dwelling older men (around 70 years on average) with lower testosterone levels are more likely to die from CV disease [11,12]. In contrast, a low testosterone level was not associated with CV deaths [19] or events [20] in community-dwelling middle-aged men (early 50s on average). These different findings might arise from the characteristics of the populations such as age and coronary risk factors, duration of follow-up and/or cut-off level of plasma testosterone at baseline. In any case, since all the above-mentioned studies were achieved in Caucasians, our study is the first to investigate the relationship between endogenous testosterone and CV events in Asians. Also, the present study showed a positive association between low testosterone level and CV events in middle-aged men with coronary risk factors, implying the clinical importance of measuring plasma testosterone in patients at risk, even if they are not old.

Unlike the previous reports showing an association of CV events with low levels of DHEA-S [21] and estradiol [22], and with a high cortisol:testosterone ratio [20], the present study did not show any significant association of CV events with estradiol, cortisol or cortisol:testosterone ratio (data not shown). The association between low DHEA-S and CV events was abolished by statistical adjustment for age, suggesting that the age-dependent decline of DHEA-S (Pearson's correlation coefficient between age and DHEA-S: −0.588; $P<0.001$) might have eliminated the association with CV events if present. Taking together with the Cox regression model including all hormones, it is suggested that testosterone is the strongest among four steroid hormones that could be predictive of CV events in this population.

There could be several mechanisms by which endogenous testosterone protects men from CV disease. Consistent with the present study, observational studies [4–8,11,12] suggest that testosterone might prevent risk factors such as obesity, hypertension, dyslipidemia, diabetes and endothelial dysfunction. Supplementary studies support the beneficial effects of testosterone on adiposity [23] and endothelial vasomotor function [24]. Based on these findings, risk markers and endothelial vasomotor function were entered into the multivariate models. Although statistical adjustment may have been insufficient to exclude the interaction between testosterone and these risk factors, testosterone remained a significant predictor of CV events in the present study. Testosterone has been reported to inhibit vascular smooth muscle cell proliferation and neointima formation [25], suggesting the direct action of testosterone on the vasculature. Also, the effects of testosterone on inflammation, hemostasis and cardiac ischemia [26] might be involved in the final process leading to CV events. The precise mechanisms, including the role of the androgen receptor and aromatization to estrogen, should be addressed in the future.

The finding of this study should not be extended to men without coronary risk factors. Our preliminary data of 47 middle-aged men without coronary risk factors showed that no subject suffered CV events during the mean follow-up period of 102 months, although a quarter of them had plasma testosterone level below the cut-off of this study (<14.2 nmol/L). Thus, the relationship between plasma testosterone and CV outcomes might be totally different in middle-aged Japanese men without coronary risk factors.

This study has several limitations. First, the number of CV events was too small to reach a clear conclusion with strong statistical power, due primarily to the small sample size and secondarily to the low incidence of CV events (approximately 2%/year). Second, the largely retrospective design (the protocol had been approved a few years before the final data collection) reduced the quality of the study and compelled us to lose many plasma samples and 16 subjects in the follow-up. Third, not all the CV events were confirmed by medical recordings. Two cases (a case in the lowest tertile and another in the middle tertile of plasma testosterone level) were determined according to the phone interview to each patient. Although the exclusion of these two cases did not significantly influence the statistical results (data not shown), self-reported outcomes limit the accuracy of this study. Fourth, the potential influence of medication on plasma testosterone level and on CV events cannot be excluded, although statistical adjustment for each class of drugs did not affect the results. For instance, beta-blockers have been reported to decrease plasma testosterone [27], but were taken by only nine subjects and were not related to testosterone level in our population (data not shown). Fifth, active forms of testosterone such as bioavailable and calculated free testosterone were not measured, because a direct assay of bioavailable testosterone or an assay of sex hormone binding globulin, which is necessary for free testosterone calculation, is not available in Japan. However, since previous longitudinal studies [11,12] have shown an association of total testosterone with CV mortality, the fundamental findings might not have differed if active forms of testosterone had been analyzed.

In summary, a low plasma testosterone level was associated with CV events in middle-aged Japanese men, independent of coronary risk factors and endothelial function. This study is the first to show the relationship between endogenous testosterone and CV events in Asian population, and provides evidence supporting the protective role of endogenous testosterone in the development of CV disease in men.

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