Table 3 Changes in mobility and self-care scores in Barthel Index during the study

Domains (points)	Mean ± SD Baseline	3 months	6 months	Change (0–6 months)	*P*
Mobility (55)					
DHEA	46.9 ± 9.2	48.2 ± 6.0	49.2 ± 5.2	2.3 ± 5.4	0.01
Control	47.5 ± 5.4	46.2 ± 5.5	45.0 ± 4.3*	−3.7 ± 3.9	
Self care (45)					
DHEA	42.7 ± 6.1	44.5 ± 1.5	43.1 ± 2.5	0.4 ± 6.9	0.96
Control	41.8 ± 4.2	42.5 ± 3.4	41.2 ± 4.3	0.7 ± 3.2	

Mobility is the sum score of five domains: (i) transfer (moving from a bed to a wheelchair and back); (ii) walking on a level surface; (iii) propelling a wheel chair; (iv) ascending and descending stairs; and (v) bathing and toilet use. Self care includes feeding, grooming, dressing, bowels and bladder. *P*-values are for repeated-measure ANOVA over all three time points. $^{*}P < 0.05$ compared to baseline. SD, standard deviation.

Table 4 Changes in cognitive domain scores during study

Domains (points)	Mean ± SD Baseline	3 months	6 months	Change (0–6 months)	*P*
Orientation (10)					
DHEA	8.3 ± 1.9	8.0 ± 2.7	7.5 ± 3.0	−0.1 ± 1.2	0.28
Control	8.3 ± 1.9	8.0 ± 2.8	7.5 ± 2.9	−0.7 ± 1.7	
Verbal memory (9)					
DHEA	5.7 ± 2.1	6.5 ± 2.3	6.7 ± 2.5†	1.0 ± 1.9	0.79
Control	6.5 ± 1.7	7.5 ± 1.8	7.0 ± 1.9	0.5 ± 1.7	
Attention and calculation (5)					
DHEA	2.3 ± 1.9	2.8 ± 2.0	2.7 ± 1.8	0 ± 2.3	0.79
Control	2.0 ± 1.7	1.9 ± 1.2	1.8 ± 1.5	−0.5 ± 1.4	
Visual memory (5)					
DHEA	3.6 ± 0.9	3.6 ± 1.3	3.8 ± 1.2	0.3 ± 1.1	0.91
Control	3.6 ± 1.3	3.9 ± 0.9	3.9 ± 1.0	0.5 ± 1.1	
Language comprehension (9)					
DHEA	8.5 ± 0.8	7.8 ± 2.5	8.7 ± 0.7	0.1 ± 0.3	0.12
Control	8.5 ± 0.8	8.5 ± 0.8	8.4 ± 1.1	−0.1 ± 0.9	
Verbal fluency (5)					
DHEA	2.8 ± 3.3	2.5 ± 2.0	4.3 ± 1.1*	1.5 ± 1.7	0.01
Control	3.2 ± 1.9	3.8 ± 1.6	3.3 ± 1.9	0.1 ± 2.1	
Performance (7)					
DHEA	5.7 ± 0.7	5.5 ± 0.7	4.8 ± 0.4**	−0.8 ± 0.6	0.36
Control	5.6 ± 0.6	5.1 ± 0.6	4.5 ± 0.9**	−1.1 ± 0.8	

Change refers to score change during 0–6 months for each parameter in each treatment group. *P*-values are for repeated-measure ANOVA over all three time points. DHEA, dehydroepiandrosterone. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\dagger}P < 0.1$ vs baseline. SD, standard deviation.

function is unknown. In the present study, plasma estradiol level was not significantly increased after DHEA treatment, implying that its beneficial effects on cognition might be androgen-dependent. Unfortunately, free testosterone levels were not measured, because they were considered to be undetectable in many cases in older women. In addition, sex hormone-binding globulin (SHBG) measurement was not available; however, it has been reported that DHEA 50 mg treatment for 3 months in postmenopausal women did not significantly change SHBG levels,[39] suggesting that the change in SHBG-bound hormone levels after DHEA treatment might be minimal. Given the local aromatization of androgen to estradiol in the brain, the effect of DHEA on cognition might be indirect, complex and heterogeneous. The molecular mechanism underlying the association

between DHEA and cognitive function needs to be clarified, and active forms of testosterone and estradiol should also be examined to investigate whether they would change after DHEA administration.

In our previous study, plasma DHEA and DHEA-S levels were independently related to higher basic ADL in older women aged 70–93 years with functional decline,[21] and other reports have shown a correlation between DHEA level and muscle mass, strength and physical performance.[40,41] In the present study, DHEA treatment maintained the Barthel Index score, while the score deteriorated significantly in the control group. Regarding body composition and strength, DHEA administration in postmenopausal older women aged up to 80 years did not alter body composition, physical performance or strength.[18–20] However, in one small-scale open-label trial, DHEA treatment for 4 weeks improved ADL in three out of seven patients (both men and women) with multi-infarct dementia.[42] All these studies are preliminary, and large-scale and long-term studies are required to ascertain whether DHEA could have a beneficial effect on ADL in older women.

In the present study, no effect of DHEA on depressive mood or vitality was observed, consistent with most clinical trials in older women.[15,43,44] This might be attributable to the participants' relatively low depressive status and high vitality status, namely, ceiling effects.

The limitations of our study should be acknowledged. First, this study was neither blinded nor randomized. Second, the number of participants was too small to confirm the results. Thus, results need to be confirmed by large-scale randomized trials to exclude possible selection bias. Third, considering the sensitivity and accuracy, a standard test like the Alzheimer's Disease Assessment Scale should be used in clinical trials to ascertain the effect of DHEA. Finally, our study duration was 6 months so it does not provide any information on the effects of longer-term DHEA supplementation.

In summary, this small study showed that supplementation of DHEA 25 mg for 6 months to older women with mild to moderate cognitive impairment improved cognitive scores and maintained basic ADL. The results should be confirmed in large-scale randomized trials.

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ORIGINAL ARTICLE

Association of low testosterone with metabolic syndrome and its components in middle-aged Japanese men

Masahiro Akishita[1], Shiho Fukai[1], Masayoshi Hashimoto[2], Yumi Kameyama[1], Kazushi Nomura[1], Tetsuro Nakamura[3], Sumito Ogawa[1], Katsuya Iijima[1], Masato Eto[1] and Yasuyoshi Ouchi[1]

Epidemiological studies have shown that low testosterone is associated with metabolic syndrome (MetS) in Caucasian men. We investigated whether testosterone level is related to the prevalence of MetS in middle-aged Japanese men. A cross-sectional survey was conducted in 194 men aged 30–64 years (49 ± 9). Blood sampling was performed in the morning after a 12-h fast, and the relationship between plasma hormone and MetS was analyzed. Low total testosterone was associated with MetS according to the Japanese criteria (HRs of 2.02 by quartile of testosterone; 95% CI=1.43–2.87) and the International Diabetes Federation criteria (HRs of 1.68 by quartile of testosterone; 95% CI=1.25–2.25). Age-adjusted regression analyses revealed that testosterone was significantly related to the MetS parameters of obesity (β=−0.365 and −0.343 for waist circumference and body mass index, respectively), hypertension (β=−0.278 and −0.157 for systolic and diastolic blood pressure, respectively), dyslipidemia (β=−0.242 and 0.228 for triglycerides and high-density lipoprotein cholesterol, respectively), insulin resistance (β=−0.253 and −0.333 for fasting plasma glucose and homeostasis model assessment of insulin resistance, respectively) and adiponectin (β=0.216). Inclusion of waist circumference into the model largely weakened the association of testosterone with other metabolic risk factors. In contrast, high estradiol was associated with MetS and its parameters, mostly attributing to the positive correlation between estradiol and obesity. Dehydroepiandrosterone sulfate was not associated with MetS or its parameters. These results suggest that low testosterone is associated with MetS and its parameters in middle-aged Japanese men. The association between estradiol and MetS needs further investigation.
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Keywords: androgen; estrogen; insulin resistance; obesity; sex hormone

INTRODUCTION

There is growing awareness that metabolic syndrome (MetS) is one of the most important threats to public health because of its association with type 2 diabetes mellitus, cardiovascular disease and mortality.[1–3] In men, it is well established that endogenous androgens decline with advancing age,[4] and low testosterone levels have been associated with insulin resistance,[5] type 2 diabetes,[6,7] hypertension[8] and increased cardiovascular and all-cause mortality.[9,10] Moreover, men with low testosterone are likely to have more components of MetS in cross-sectional studies,[11–13] and longitudinal studies show that lower total testosterone predicts higher frequency of MetS.[14,15] These data were mostly from studies with Caucasian men in western countries. Regarding Japanese men, one study showed that testosterone was positively correlated with plasma adiponectin.[16] However, there are no reports showing a relationship between testosterone and MetS or its components in Japanese men.

Recently, we reported that low testosterone is an independent determinant of endothelial dysfunction in middle-aged men[17] and is a predictor of cardiovascular events in men with coronary risk factors,[18] suggesting a link between testosterone and cardiovascular pathology. Given these findings, this study investigated the relationship of endogenous testosterone with MetS in middle-aged Japanese men.

METHODS

Subjects

Enrollment screening included consecutive, apparently healthy male subjects aged 30–64 years who underwent medical examinations at either our department or at two clinics located in Tokyo. After exclusion of subjects who met the exclusion criteria, 194 subjects (104 from our department and 90 from the clinics) were enrolled. Exclusion criteria included history of cardiovascular disease (stroke, coronary heart disease, congestive heart failure and peripheral arterial disease), malignancy or overt endocrine disease or use of steroid hormones, because these conditions may influence plasma sex hormones and/or the components of MetS. Other exclusion criteria were diabetic subjects

[1]Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [2]Department of General Internal Medicine, Kobe University School of Medicine, Kobe, Japan and [3]Research Institute of Aging Science, Tokyo, Japan
Correspondence: Dr M Akishita, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: akishita-tky@umin.ac.jp
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on insulin injection or hypoglycemic agent drugs or with hemoglobin A1c >8%, and subjects on β-blockers[19] or fibrates. History, physical examination and laboratory tests were performed for all subjects. Of the included subjects, 23% (n=44) were taking anti-hypertensive drugs (angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers and diuretics), and 22% were taking statins. Each subject gave written, informed consent before study enrollment. The study protocol was approved by the ethics committee of the Graduate School of Medicine at the University of Tokyo.

Assays of metabolic risk factors and plasma hormones

Clinical information was collected at baseline when each patient attended the initial medical examination. Blood sampling and measurement of height, weight, waist circumference and blood pressure were performed in the morning after a 12-h overnight fast. Blood pressure was measured at least twice using an automated, digital electrosphygmomanometer (Omron Healthcare, Kyoto, Japan) on the non-dominant arm in a sitting position, and the average was used for statistical analysis.

Serum total cholesterol and triglyceride were measured enzymatically, and serum high-density lipoprotein (HDL) cholesterol was measured by the heparin-$Ca^{2+}Ni^{2+}$ precipitation method. Low-density lipoprotein cholesterol was determined using the Friedewald formula or the direct, liquid, selective detergent method when triglycerides were >400 mg per 100 ml. Plasma glucose was assayed by the glucose oxidase method, and hemoglobin A1c was measured by high-performance liquid chromatography. Plasma total testosterone, dehydroepiandrosterone sulfate and estradiol were determined using sensitive radioimmunoassays. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin ($\mu IU\,ml^{-1}$)×fasting plasma glucose (mg per 100 ml)/405. Patients with a fasting plasma glucose >140 mg per 100 ml were excluded from the HOMA-IR calculation because of a lack of data reliability. Serum adiponectin was measured using an enzyme-linked immunosorbent assay (Human Adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo, Japan). These assays were performed by a commercial laboratory (SRL, Tokyo, Japan). The intra-assay coefficients of variation for the measurements were <5%.

Definition of MetS

We applied both the Japanese criteria[20] and the International Diabetes Federation (IDF) criteria for Japanese ethnicity[21] for the diagnosis of MetS. In the Japanese criteria, MetS was diagnosed when waist circumference ≥85 cm and two or more of the following three components were present: (1) HDL cholesterol <40 mg per 100 ml and/or triglyceride ≥150 mg per 100 ml; (2) systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg and (3) fasting plasma glucose ≥110 mg per 100 ml. Subjects taking anti-hypertensive medications were considered hypertensive for statistical purposes.

In the IDF criteria for Japanese ethnicity, MetS was diagnosed when waist circumference ≥85 cm and two or more of the following four components were present: (1) HDL cholesterol <40 mg per 100 ml; (2) triglyceride ≥150 mg per 100 ml; (3) systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg and (4) fasting plasma glucose ≥100 mg per 100 ml. Subjects taking anti-hypertensive medications were considered hypertensive for statistical purposes.

Data analysis

Values are expressed as the mean ± s.d. in the text unless otherwise stated. Pearson's simple correlation coefficients were calculated between plasma hormones and the number of MetS components. Differences between the quartile groups of sex hormones were analyzed using one-factor ANOVA followed by the Newman–Keuls' test. Logistic regression analysis was performed to determine the association of sex hormones with the diagnosis of MetS. Furthermore, multiple regression analysis was performed to determine the association between sex hormones and metabolic risk factors for MetS. A value of $P<0.05$ was considered statistically significant. The data were analyzed using SPSS (Version 17.0, SPSS, Chicago, IL, USA).

RESULTS

Sex hormones and MetS criteria

Characteristics of the study subjects are shown in Table 1. Twenty-three and 32% of the subjects were diagnosed with MetS according to the Japanese criteria and the IDF criteria, respectively. The prevalence is comparable with that reported in middle-aged Japanese men.[22,23]

As plasma total testosterone was negatively correlated with the number of MetS components (Figure 1a), the association of testosterone with MetS was analyzed by quartile of testosterone. As shown in Figure 2a, lower testosterone was associated with a step-wise increase in the number of MetS components. Age-adjusted logistic regression analysis revealed that the hazard ratios for MetS diagnosis by quartile decline of testosterone were 2.02 (95% CI=1.43–2.87) and 1.68 (95% CI=1.25–2.25) according to the Japanese criteria and the IDF criteria, respectively.

Interestingly, plasma estradiol was positively correlated with the number of MetS components (R=0.285, $P<0.001$); therefore, the association with MetS was also analyzed by quartile of estradiol. As shown in Figure 2b, higher estradiol was associated with a step-wise increase in the number of MetS components. Age-adjusted logistic regression analysis revealed that the hazard ratios for MetS diagnosis by quartile increment of estradiol were 1.48 (95% CI=1.06–2.06) and 1.63 (95% CI=1.20–2.21) according to the Japanese criteria and the IDF criteria, respectively. Dehydroepiandrosterone sulfate was not associated with MetS components or diagnosis (data not shown).

Table 1 Characteristics of study subjects (N=194)

Age (years)	49 ± 9	[30–64]
Body mass index ($kg\,m^{-2}$)	25.2 ± 4.0	[17.3–41.9]
Waist circumference (cm)	87 ± 10	[69–125]
Hip circumference (cm)	96 ± 7	[80–125]
Waist/hip ratio	0.94 ± 0.06	[0.78–1.09]
Systolic blood pressure (mm Hg)	126 ± 14	[95–183]
Diastolic blood pressure (mm Hg)	79 ± 11	[50–128]
Triglycerides (mg per 100 ml)	162 ± 135	[32–880]
HDL cholesterol (mg per 100 ml)	54 ± 16	[26–110]
Free fatty acids ($mEq\,l^{-1}$)	0.53 ± 0.28	[0.08–2.08]
LDL cholesterol (mg per 100 ml)	128 ± 29	[54–213]
Fasting plasma glucose (mg per 100 ml)	98 ± 13	[76–158]
Hemoglobin A1c (%)	5.2 ± 0.6	[4.0–8.0]
Insulin ($\mu U\,ml^{-1}$)	6.7 ± 4.0	[1.0–21.2]
HOMA-IR	1.64 ± 1.04	[0.21–5.50]
Total testosterone ($nmol\,l^{-1}$)	19.1 ± 6.2	[4.6–38.2]
DHEA-S ($\mu mol\,l^{-1}$)	5.89 ± 2.37	[1.12–12.0]
Estradiol ($pmol\,l^{-1}$)	92.5 ± 43.7	[18.4–216.6]
Metabolic syndrome (MetS) and its components		
MetS (Japanese criteria), n (%)		44 (23)
MetS (IDF criteria), n (%)		62 (32)
Waist circumference ≥85 cm, n (%)		110 (56)
High blood pressure, n (%)		89 (46)
HDL cholesterol <40 mg per 100 ml, n (%)		34 (18)
Triglycerides ≥150 mg per 100 ml, n (%)		79 (41)
Fasting plasma glucose ≥110 mg per 100 ml, n (%)		23 (12)
Fasting plasma glucose ≥100 mg per 100 ml, n (%)		73 (38)

Abbreviations: DHEA-S, dehydroepiandrosterone sulfate; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IDF, International Diabetes Federation; LDL, low-density lipoprotein.
Values are expressed as the mean ± s.d. (range). High blood pressure was defined if subjects showed systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg, or were taking antihypertensive medications.

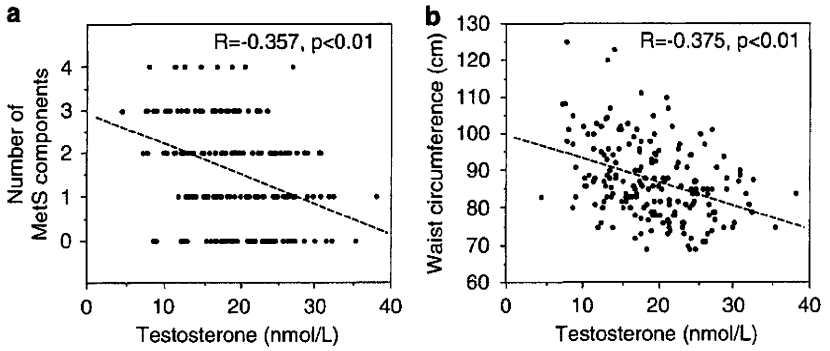

Figure 1 Scattergrams and regression lines (dotted lines) showing the correlation between testosterone and the number of metabolic syndrome (MetS) components (**a**) or waist circumference (**b**).

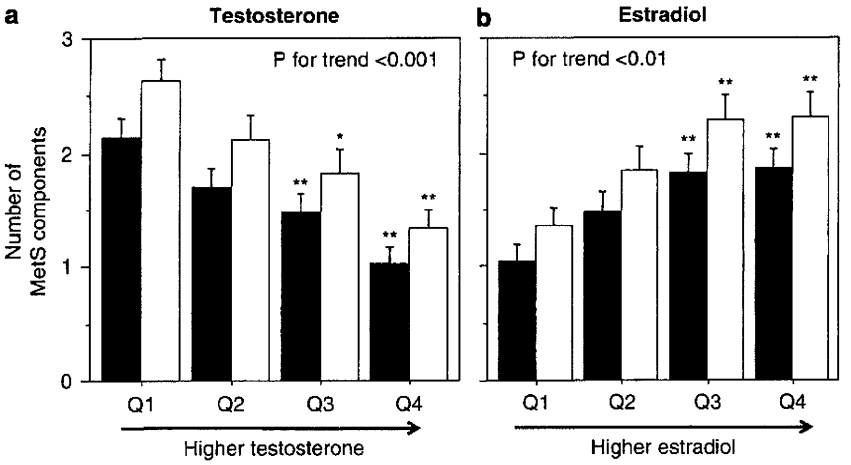

Figure 2 Number of metabolic syndrome (MetS) components according to quartiles of plasma testosterone (**a**) and estradiol (**b**). MetS components were defined according to the Japanese criteria (closed bars) and the IDF criteria for Japanese ethnicity (open bars). Values are expressed as the mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* Q1. Cut offs of the quartiles were 14.1, 18.7 and 23.4 nmol l^{-1} (405, 540 and 674 ng per 100 ml) for testosterone, and 55, 101 and 125 pmol l^{-1} (15.0, 27.5 and 34.0 pg ml^{-1}) for estradiol.

Sex hormones and metabolic risk factors

The associations of plasma sex hormones with each of the metabolic risk factors were analyzed. As shown in Table 2, the unadjusted model shows that testosterone was significantly related to parameters of MetS except for diastolic blood pressure. Testosterone was not related to low-density lipoprotein cholesterol, but this parameter is not included in the definitions of MetS used here. Adjustment for age did not considerably influence the results of the regression analysis, but the association between testosterone and diastolic blood pressure became significant after adjustment for age. In contrast, inclusion of waist circumference into the model weakened the association of testosterone with metabolic risk factors. As a result, systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR were significantly related to testosterone. The significant association for diastolic blood pressure, HDL cholesterol, free fatty acids, hemoglobin A1c, insulin and adiponectin were attenuated after adjustment for age and waist circumference. Adjustment for body mass index or waist/hip ratio instead of waist circumference showed similar results (data not shown).

As shown in Table 3, estradiol showed weaker association than testosterone with parameters of MetS, but was significantly related to body mass index, waist circumference, systolic blood pressure, HDL

Table 2 Multiple regression analysis determining the impact of plasma testosterone on metabolic risk factors

	Unadjusted	*Age adjusted*	*Age+waist adjusted*
Body mass index	−0.376*	−0.343*	ND
Waist circumference	−0.378*	−0.365*	ND
Waist/hip ratio	−0.353*	−0.384*	ND
Systolic blood pressure	−0.230**	−0.278*	−0.169***
Diastolic blood pressure	−0.114	−0.157***	−0.098
Triglycerides	−0.247*	−0.242*	−0.182***
HDL cholesterol	0.252*	0.228**	0.065
Free fatty acids	−0.208**	−0.209**	−0.137
LDL cholesterol	−0.054	−0.056	−0.020
Fasting plasma glucose	−0.231**	−0.253**	−0.228**
Hemoglobin A1c	−0.166***	−0.220**	−0.137
Insulin	−0.331*	−0.307*	−0.129
HOMA-IR	−0.349*	−0.333*	−0.159***
Adiponectin	0.222**	0.216**	0.046

Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; ND, not determined.
Regression coefficients with plasma testosterone as an independent variable and each of risk factors as a dependent variable are shown. Age and/or waist circumference were included in multiple regression models as indicated. $^{*}P<0.001$, $^{**}P<0.01$, $^{***}P<0.05$.

Table 3 Multiple regression analysis determining the impact of plasma estradiol on metabolic risk factors

	Unadjusted	*Age adjusted*	*Age+waist adjusted*
Body mass index	0.279*	0.260*	ND
Waist circumference	0.346*	0.338*	ND
Waist/hip ratio	0.102	0.082	ND
Systolic blood pressure	0.133	0.158**	0.042
Diastolic blood pressure	0.036	0.058	−0.002
Triglycerides	0.105	0.094	−0.012
HDL cholesterol	−0.207***	−0.193***	−0.040
Free fatty acids	0.087	0.091	0.049
LDL cholesterol	−0.056	−0.056	−0.094
Fasting plasma glucose	0.130	0.141	0.095
Hemoglobin A1c	0.040	0.067	−0.030
Insulin	0.240***	0.228***	0.038
HOMA-IR	0.250***	0.243***	0.060
Adiponectin	−0.267*	−0.262*	−0.114

Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; ND, not determined.
Regression coefficients with plasma estradiol as an independent variable and each of risk factors as a dependent variable are shown. Age and/or waist circumference were included in multiple regression models as indicated. *$P<0.001$, **$P<0.05$, ***$P<0.01$.

cholesterol, insulin, HOMA-IR and adiponectin after adjustment for age. Further adjustment for waist circumference, body mass index or waist/hip ratio (Table 3 and data not shown) eliminated the significant associations between estradiol and these metabolic parameters. Dehydroepiandrosterone sulfate was not significantly related to parameters of MetS in unadjusted or adjusted analyses (data not shown).

DISCUSSION

In this study, cross-sectional analysis of 194 middle-aged Japanese men showed that low testosterone is positively related to MetS, MetS components and additional metabolic risk factors. Adjustment for obesity parameters such as waist circumference, body mass index and waist/hip ratio greatly diminished the association, but low testosterone retained weak associations with some metabolic risk factors including systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR. Taken together, results in this statistical model suggest that abdominal obesity is an important contributor to the association between low testosterone and MetS, but additional factors may also impact testosterone. To our knowledge, this is the first report showing the significant association between low testosterone and MetS in Japanese men.

Several mechanisms have been suggested for the causal relationship between low testosterone and abdominal obesity. Activation of the lipoprotein lipase and lipolysis[24] may explain the effect of testosterone on adipose tissue. Many studies including a medium-sized, randomized controlled trial[25] and a meta-analysis[26] showed the inverse effect of testosterone on adiposity. Conversely, it has been reported that men with MetS are prone to hypogonadism.[27] This finding might be due to elevated leptin levels that interfere with gonadotropin-stimulated androgen production[28] and to increased aromatase activity in adipose tissue that leads to higher circulating estradiol and suppression of testosterone production by negative feedback.[29] These findings suggest a bi-directional causal relationship between low testosterone and obesity.

After adjustment for waist circumference, testosterone was weakly but significantly related to some metabolic risk factors including systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR, which is consistent with earlier reports.[5,6,8,12] Testosterone is likely to be involved in the pathogenesis of MetS, irrespective of obesity. For example, testosterone increases the hepatic production of apolipoprotein A-1 and consequently increases HDL cholesterol,[30] improves insulin sensitivity and increases muscle strength.[31] There was no significant correlation between age and testosterone ($R=0.114$, $P=0.12$). This result may be because the cohort was limited to middle-aged men (30–64 years old) . However, age was included in the multivariate analyses in this study, because it is well established that testosterone declines with age.[4]

The positive association found between testosterone and adiponectin is in agreement with earlier reports.[16,32,33] However, the direct action of testosterone on adiponectin production/secretion might be different from these findings, because testosterone decreases adiponectin secretion in mice and in adipocytes.[34,35] Accordingly, abdominal obesity may underlie the positive correlation between testosterone and adiponectin in men.

In this study, estradiol was associated positively with MetS and its components, consistent with an earlier report.[12] This relationship may be independent of testosterone because estradiol was not correlated with testosterone by simple regression analysis ($R=-0.019$, $P=0.80$), and the inclusion of both testosterone and estradiol into the multiple regression model as covariates did not influence the association of each other with MetS parameters (data not shown). The relationship between estradiol and MetS might be attributed to increased aromatase activity and subsequent elevation of circulating estradiol in obese subjects.[29] Increased estradiol may subsequently suppress pituitary function,[29] and lead to a further decrease in testosterone. Comprehensive assessment of sex hormone, gonadotropin and components of MetS reveal a causal relationship. Unfortunately, we could not measure gonadotropin because of limited plasma. Further investigation is needed to address the mechanistic and pathophysiological interactions between sex hormones and MetS.

There are some limitations to our study. First, the cross-sectional design does not clarify the causal relationship between sex hormones and MetS. As there may be bi-directional causalities as mentioned above, longitudinal follow-up studies and hormone replacement studies should be performed in Japanese populations. Second, active forms of testosterone such as bioavailable and calculated free testosterone were not measured. A direct assay of bioavailable testosterone or of sex hormone-binding globulin (required for free testosterone calculation) was not available for the study. Third, the potential influence of medications on the measured parameters cannot be denied, although the exclusion of subjects on statins ($n=40$) or anti-hypertensive drugs ($n=44$) did not seriously affect the association of testosterone with waist circumference (statins, $R=-0.304$, $P<0.01$; anti-hypertensives, $R=-0.337$, $P<0.01$) and the number of MetS components (statins, $R=-0.274$, $P<0.01$; anti-hypertensives, $R=-0.278$, $P<0.01$). Fourth, because the sample size ($n=194$) is relatively small, the finding needs to be confirmed in a larger cohort.

In summary, this study suggests that low testosterone is associated with MetS and its parameters in middle-aged Japanese men. We also found a positive but weaker association between estradiol and MetS. These associations were largely attenuated by adjustment for waist circumference. Our results reinforce the need to address the causal relationship and pathophysiological interactions between sex hormones and MetS.

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

Jing Yu, Masahiro Akishita, Masato Eto, Sumito Ogawa, Bo-Kyung Son, Shigeaki Kato, Yasuyoshi Ouchi, and Tetsuro Okabe

Departments of Integrated Traditional Medicine (J.Y., T.O.) and Geriatric Medicine (M.A., M.E., S.O., B.-K.S., Y.O.), Graduate School of Medicine, and the Institute of Molecular and Cellular Bioscience (S.K.), The University of Tokyo, Tokyo 113-8655, Japan

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-

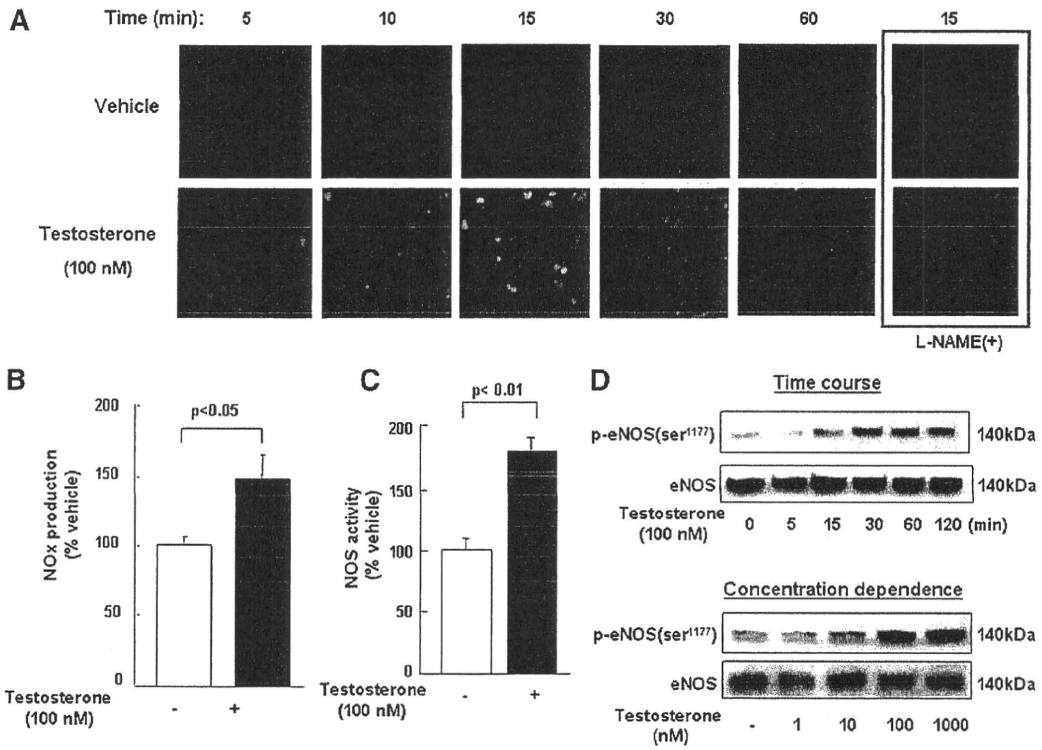

FIG. 1. Testosterone rapidly stimulates NO production and NOS activation in HAEC. A, Growth-arrested HAEC were loaded with DAF-2DA before treatment with or without testosterone for the indicated times. After testosterone treatment, cells were fixed in 2% paraformaldehyde and viewed using a fluorescence 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

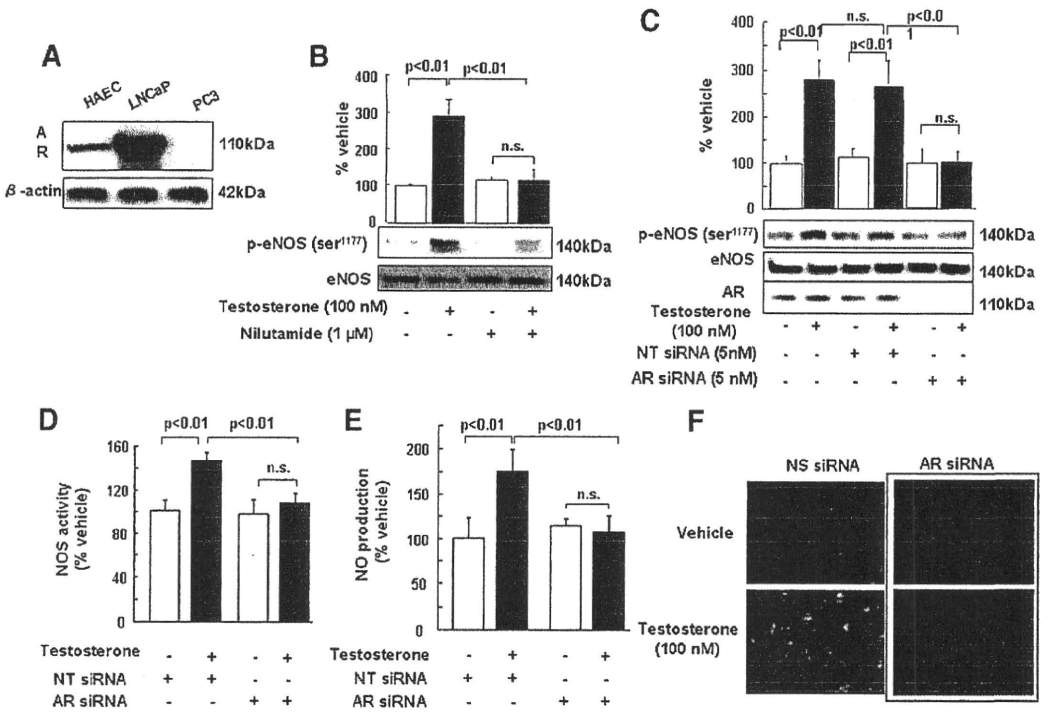

FIG. 2. AR is involved in testosterone-induced eNOS phosphorylation. A, AR expression was examined in HAEC, AR-positive LNCaP cells, and AR-negative PC3 cells using immunoblotting (*upper panel*). The membrane was stripped and re-immunoblotted with anti-β-actin antibody for monitoring equal amounts of 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.

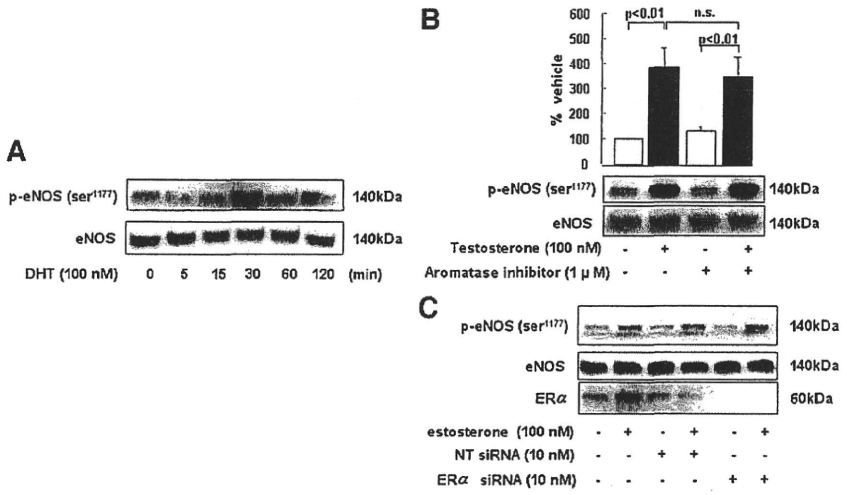

FIG. 3. Estradiol/ER pathway is not involved in testosterone-induced eNOS phosphorylation. A, Growth-arrested HAEC were treated with DHT or vehicle for the indicated times. B, Growth-arrested cells were treated with testosterone or vehicle for 30 min in the presence or absence of aromatase inhibitor. 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).

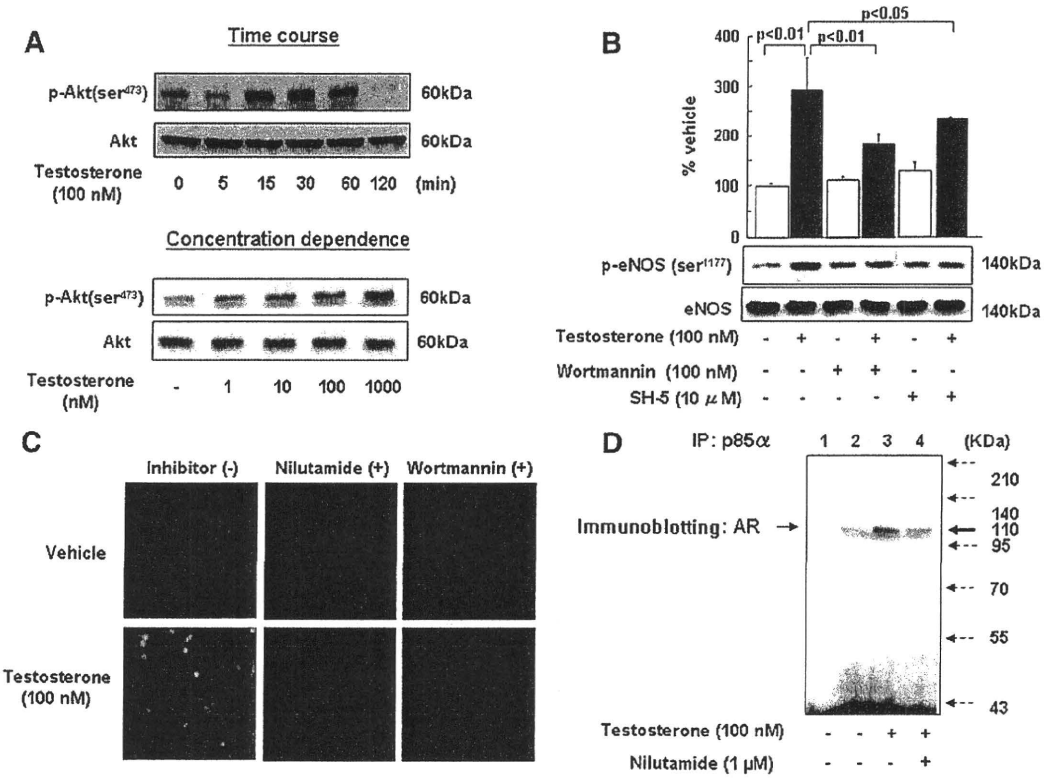

FIG. 4. PI3-kinase/Akt pathway is involved in testosterone-induced eNOS phosphorylation and NO production. A, Growth-arrested HAEC were treated with vehicle or testosterone for the indicated times or with the indicated concentrations of testosterone for 30 min. Phosphorylation of Akt at Ser473 (p-Akt) 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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Address all correspondence and requests for reprints to: Masahiro Akishita, M.D., Ph.D., Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo; 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: akishita-tky@umin.ac.jp.

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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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Bo-Kyung Son[‡], Masahiro Akishita[‡1], Katsuya Iijima[‡], Sumito Ogawa[‡], Koji Maemura[§], Jing Yu[¶], Kenichi Takeyama[‖], Shigeaki Kato[‖], Masato Eto[‡], and Yasuyoshi Ouchi[‡]

From the [‡]Department of Geriatric Medicine and the [¶]Department of Integrated Traditional Medicine, the Graduate School of Medicine and the [‖]Institute of Molecular and Cellular Biosciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-8655 and the [§]Department of Cardiovascular Medicine Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8501, Japan

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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[1] To whom correspondence should be addressed: Dept. of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: 81-3-5800-8832; Fax: 81-3-5800-8831; E-mail: akishita-tky@umin.ac.jp.

[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.

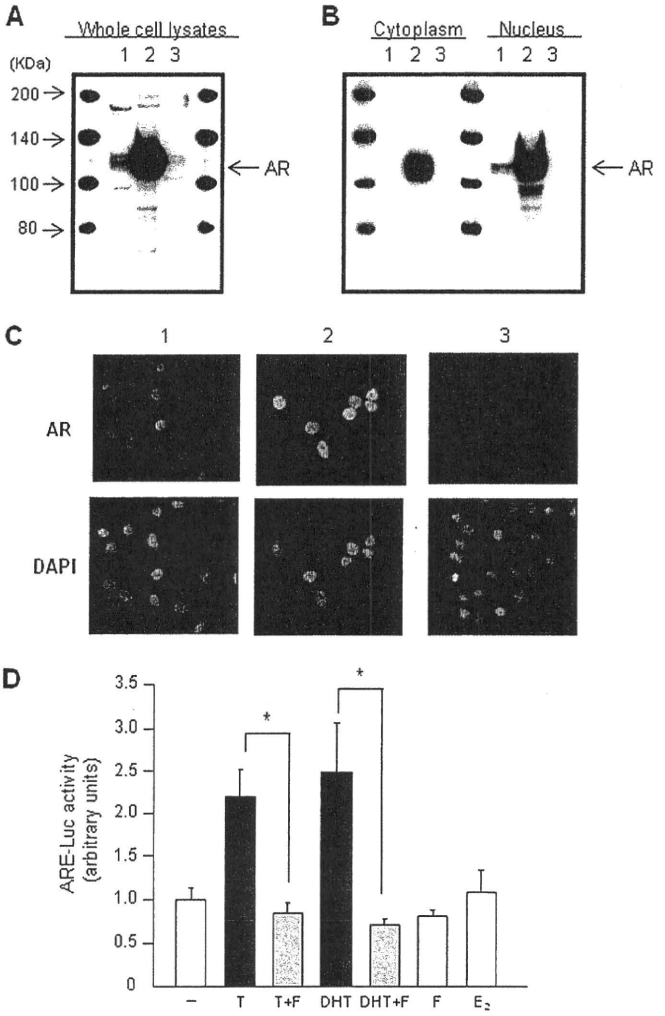

FIGURE 1. **Expression of the functional the AR in HASMC.** Endogenous expression of the AR in HASMC (*lane 1*) was examined in whole cell lysates (*A*) and cytoplasmic and nuclear fractions (*B*) compared with that in human prostate cancer cell lines, LNCaP (AR-positive; *lane 2*) and PC-3 (AR-negative;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

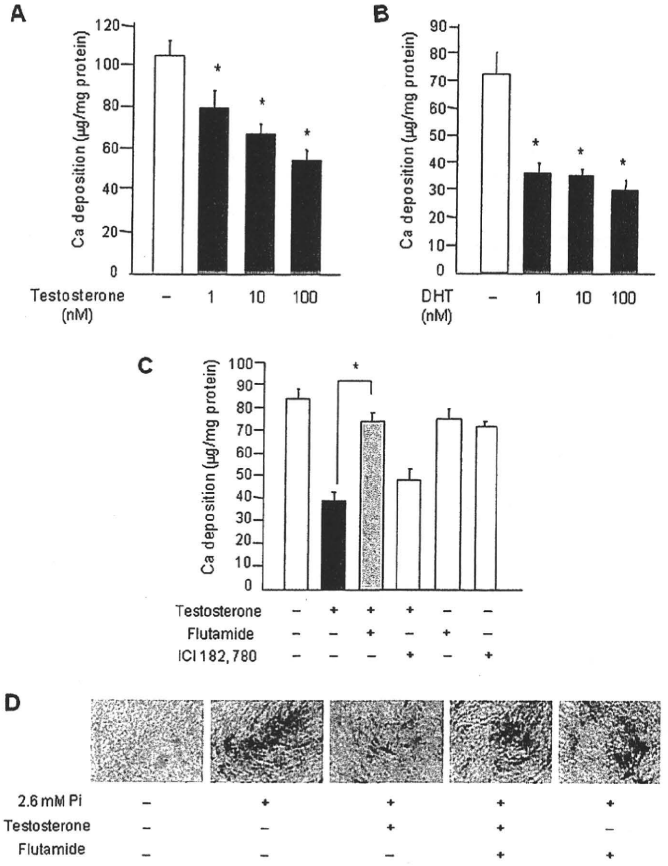

FIGURE 2. **Androgens prevent P_i-induced calcification via the AR.** HASMC were cultured with the indicated concentrations of androgens (testosterone (*A*) and DHT (*B*)) in the presence of 2.6 mM P_i for 6 days. Calcium deposition was measured by the *o*-cresolphthalein complexone method and normalized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

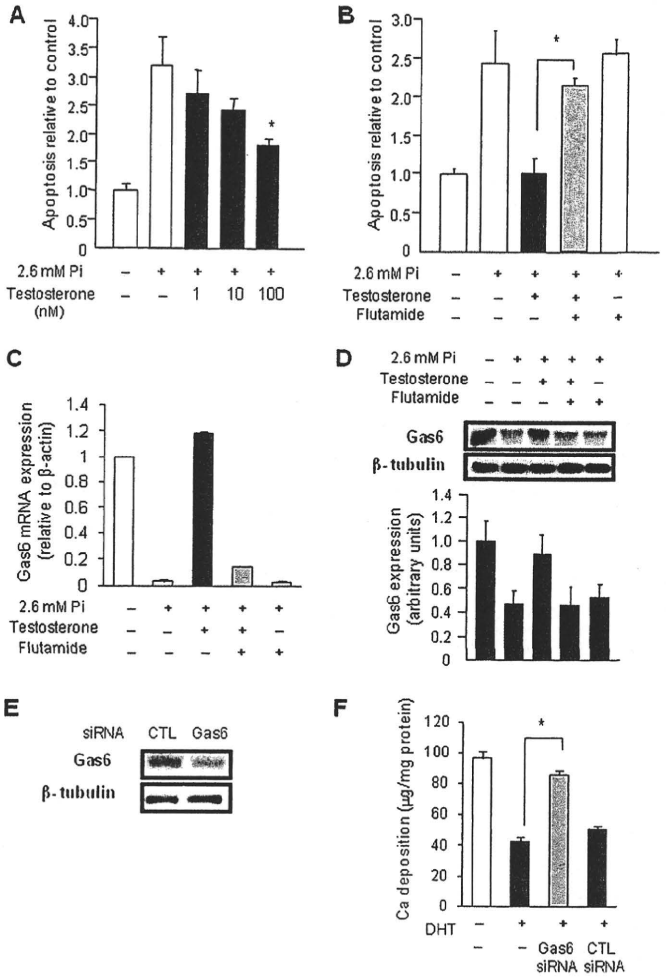

FIGURE 3. **Androgens inhibit P_i-induced apoptosis and restore Gas6-mediated survival pathway.** *A*, HASMC were cultured with the indicated concentrations of testosterone in the presence of 2.6 mM P_i for 6 days. A quantitative index of apoptosis, determined by DNA fragmentation enzyme-linked immunosorbent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