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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DHEA attenuates PDGF-induced phenotypic proliferation of vascular smooth muscle A7r5 cells through redox regulation ☆

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ABSTRACT

It is known that dehydroepiandrosterone (DHEA) inhibits a phenotypic switch in vascular smooth muscle cells (VSMC) induced by platelet-derived growth factor (PDGF)-BB. However, the mechanism behind the effect of DHEA on VSMC is not clear. Previously we reported that low molecular weight-protein tyrosine phosphatase (LMW-PTP) dephosphorylates PDGF receptor (PDGFR)-β via a redox-dependent mechanism involving glutathione (GSH)/glutaredoxin (GRX)1. Here we demonstrate that the redox regulation of PDGFR-β is involved in the effect of DHEA on VSMC. DHEA suppressed the PDGF-BB-dependent phosphorylation of PDGFR-β. As expected, DHEA increased the levels of GSH and GRX1, and the GSH/GRX1 system maintained the redox state of LMW-PTP. Down-regulation of the expression of LMW-PTP using siRNA restored the suppression of PDGFR-β-phosphorylation by DHEA. A promoter analysis of GRX1 and γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme of GSH synthesis, showed that DHEA up-regulated the transcriptional activity at the peroxisome proliferator-activated receptor (PPAR) response element, suggesting PPARα plays a role in the induction of GRX1 and γ-GCS expression by DHEA. In conclusion, the redox regulation of PDGFR-β is involved in the suppressive effect of DHEA on VSMC proliferation through the up-regulation of GSH/GRX system.

1. Introduction

Vascular smooth muscle cells (VSMC), the contractile component of blood vessels, play a critical role in the pathogenesis of atherosclerosis. VSMC express a set of smooth muscle-specific genes, which are characteristic of their contractile, differentiated phenotype [1]. VSMC undergo phenotypic modulation in response to environmental signals. Accelerated migration, proliferation, and production of extracellular matrix components by phenotypically modulated VSMC play a central role in the development of atherosclerotic lesions [1].

Platelet-derived growth factors (PDGFs) bind to two cell-surface receptor-tyrosine kinases, PDGF receptor (PDGFR) α and β [2]. PDGF-dependent activation of receptors causes a mitogenic signal transduction through the phosphorylation of specific tyrosines in the receptors [2–4].

Dehydroepiandrosterone (DHEA) and the sulfated prohormone of DHEA circulate at higher plasma concentrations than any other steroids. Both the occurrence and the clinical manifestation of coronary atherosclerosis have been inversely correlated with plasma levels of DHEA or DHEA sulfate [5]. DHEA has a wide variety of beneficial biological and physiological effects on the prevention of cardiovascular disease [6].

The redox status of sulfhydryl groups in proteins plays an important role in the regulation of cellular functions such as the synthesis and folding of proteins and regulation of the structure and activity of enzymes, receptors, and transcription factors [7]. Glutaredoxin (GRX), a glutathione (GSH)-dependent oxidoreductase, catalyzes the reduction of protein disulfide via a disulfide exchange reaction [8]. Previously, we reported that GRX1 plays an important role in regulating PDGF-BB-dependent signals through down-regulation of the tyrosine phosphorylation of PDGFR-β [3]. The GSH/GRX1 system suppresses the PDGF-BB-induced tyrosine phosphorylation of PDGFR-β, resulting in suppression of the PDGF-BB-dependent cell proliferation. Furthermore, we found a novel regulatory mechanism for PDGF-BB signaling involving the redox-dependent regulation of low molecular weight-protein tyrosine phosphatase (LMW-PTP) by GRX1 in a GSH-dependent manner [3]. Recently, we also reported

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that estradiol potentiates GSH/GRX1 redox potential in cardiomyocytes through up-regulation of the gene expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis and GRX1 [9]. Then, we were interested in whether DHEA has any effects on the GSH/GRX1 system in VSMC exposed to PDGF-BB. In the present study, we demonstrate that DHEA attenuates PDGF-BB-induced VSMC proliferation and phenotypic modulation. Importantly, we show that DHEA increases the expression of GRX and GSH synthesis. This increase in GSH/GRX1 redox potential stimulates the LMW-PTP to down-regulate the activity for tyrosine phosphorylation of PDGFR-β.

2. Materials and methods

2.1. Reagents

Rabbit antibodies against PDGFR-β and phospho (Tyr-751)-PDGFR-β were obtained from Cell Signaling Technology. PDGF-BB, GSH, GSSG, NADPH, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), flutamide, and *p*-nitrophenyl phosphate were from Sigma Chemical Co. DHEA, hydrogen peroxide, and dithiothreitol (DTT) were from Wako Pure Chemicals (Osaka, Japan). Normal goat, rabbit, and mouse IgG were from Sigma Chemical Co. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was purchased from Molecular Probes. ICI182,780 was from Tocris (Ballwin, MO).

2.2. Cell culture and proliferation

Rat embryonic thoracic aorta smooth muscle-derived A7r5 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured as described [10]. Briefly, cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After the attainment of confluency (70–80%), the cells were incubated in serum-free DMEM containing 0.2% bovine serum albumin for 20–24 h. The proliferation of cultured cells was evaluated by measuring attached live cells photometrically after staining with crystal violet. A7r5 cells incubated in the presence or absence of DHEA were placed in 100 μl of medium/well in 96-well plates and cultured in medium containing 0.2% BSA with or without 0.5 nM PDGF-BB for specific periods. Then the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.5), washed, and stained with 0.01% crystal violet at room temperature for 20 min. Each well was extensively washed with water and dried. The stained cells were lysed by adding 100 μl of lysis buffer A (10% SDS and 0.1 N HCl), and the cell number was then estimated photometrically by measuring the absorbance at 570 nm using a microplate reader.

2.3. Purification of recombinant LMW-PTP and generation of antibody against LMW-PTP

LMW-PTP was purified with the glutathione *S*-transferase (GST) gene fusion system (Amersham Biosciences) according to the manufacturer's instructions. In brief, *Escherichia coli* strain BL21 cells were transformed with pGEX6p-LMW-PTP, and protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). GST-fused LMW-PTP (GST-LMW-PTP) was affinity purified from cell lysates using glutathione–Sepharose 4B (Amersham Biosciences), and then digested with PreScission protease. The cleaved GST was removed from the glutathione–Sepharose 4B, and LMW-PTP was purified. The LMW-PTP was used to immunize rabbits to generate anti-LMW-PTP antibodies, and also used for experiments concerning the redox regulation of LMW-PTP as described below.

2.4. Immunoblot analysis

Cultured cells were harvested and lysed for 20 min at 4 °C in lysis buffer. The supernatants obtained by centrifugation of the lysates at 8000*g* for 15 min were used in subsequent experiments. Protein concentrations were determined using a BCA assay kit (Pierce). Protein samples were electrophoresed on 10%, 12.5% or 15% SDS–polyacrylamide gels under reducing conditions, except for thiol-modified protein samples. The proteins in the gels were transferred onto nitrocellulose membranes, and were incubated with horseradish peroxidase-conjugated anti-IgG antibodies. Proteins in the membranes were visualized using the enhanced chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions.

2.5. Determination of redox states

The redox states of proteins were assessed by modifying free thiol with AMS [11]. Briefly, after incubation with or without PDGF-BB, cell lysates or proteins were treated with trichloroacetic acid at a final concentration of 7.5% to denature and precipitate the proteins as well as to avoid any subsequent redox reactions. The protein precipitates were collected by centrifugation at 12,000*g* for 10 min at 4 °C. The pellets were rinsed in acetone and centrifuged twice, then, dissolved in a buffer containing 50 mM Tris–HCl (pH 7.4), 1% SDS and 15 mM AMS. Proteins were then separated by 10% SDS–PAGE without using any reducing agents and blotted to nitrocellulose membranes. Proteins in the membranes were treated with 5% (w/v) nonfat dry milk and 0.1% Tween 20 in TBS solution for 1 h at room temperature, then further kept overnight at 4 °C for visualization by immunoblotting as described above.

2.6. Determination of cellular glutathione levels

GSH and glutathione disulfide (GSSG) levels were measured as described previously [9] using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Inc., MD) according to the manufacturer's directions.

2.7. Generation of luciferase reporter constructs

A 2.0-kb fragment of the human GRX1 gene promoter (−2023 to −22) was amplified by PCR using Pfu turbo DNA polymerase (Stratagene). The primers used were /5′-GGA CTG AGT GAG AGG CAG ACA ATA GTC TCC-3′/ as a forward primer, and /5′-CGG GAA GAA TCC TCA GTT GCA GGT ATT GCT TGG-3′/ as a reverse primer. The PCR product was subcloned into pUC18 to obtain pUC18-pro-GRX. PUC18-pro-GRX was digested with HindIII, and the resulting fragment containing the promoter region from −2023 to −22 was inserted into the HindIII site of the reporter vector pGL3-Basic (Stratagene) to give pGL3-pro-GRX. To generate a deleted form of the luciferase reporter construct (pGL3-pro-GRX-del), pGL3-pro-GRX was digested with KpnI and PvuII (Takara Biomedicals). Site-directed mutagenesis for luciferase vectors was performed with pGL3-pro-GRX (−2023 to −22) as a template by using a QuickChange site-directed mutagenesis kit (Stratagene). The oligonucleotides used were; peroxisome proliferator-activated receptor (PPAR) response element (PPRE)-like 1 forward (5′-GGT CAG GAT ACC TAG CTA AAT ttT CAT TTG GTG AXA TAG AGG CCA TG-3′), and PPRE-like 1 reverse (5′-CAT GGC CTC TAT GTC ACC AAA TGA aaA TTT AGC TAG GTA TCC TGA CC-3′). The nucleotide sequence was confirmed by sequencing with an ALFexpress II system (Amersham Biosciences). We constructed a 50-bp chimeric promoter with two copies of synthesized fragment of the human γ-GCS heavy subunit gene promoter (−10,625 to −10,613 bp, GenBank Accession No. AL033397) containing a PPRE-like domain; AGATCACAGGTCA. It was annealed using a forward sequence

(5′-gac ggt acc AGA TCA CAG GTC ATT GAT AAG ATC ACA GGT Cag tgg agc tc-3′) and a reverse sequence (5′-gag ctc cac TGA CCT GTG ATC TTA TCA ATG ACC TGT GAT CTg gta ccg tc-3′). The annealed product was subcloned into pUC18 to obtain pUC18-pro-γ-GCS. pUC18-pro-γ-GCS was digested with KpnI and SacI. The resulting fragment containing the promoter region (−10625 to −10613) was inserted into the HindIII site of the reporter vector pGL3-Basic to give pGL3-pro-γ-GCS. To generate a deleted version of the luciferase reporter construct (pGL3-pro-γ-GCS-del), pGL3-pro-γ-GCS was digested with KpnI and PvuII. Site-directed mutagenesis for luciferase vectors was performed with pGL3-pro-γ-GCS as a template.

2.8. Luciferase activity assay

Each vector was introduced into A7r5 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The luciferase activity was assayed with cellular extracts by using a luciferase reporter assay system (Promega).

2.9. Quantitative RT-PCR

Quantitative RT-PCR was performed using the One Step SYBR® RT-PCR kit (Perfect Real Time, TAKARA BIO INC. Japan) according to the manufacturer's directions. After the RT-PCR using Mx3000P (Stratagene), the products were analyzed using SMxProTM Software version 3.00 (Stratagene). A 764-base-pair (bp) DNA γ-GCS heavy subunit cDNA was obtained by digesting a fragment (bp 865–1628) with PstI. The 330-bp oligonucleotides for GRX1 (rat GRX sequence, Accession No. AF167981) were obtained using as a forward primer, 5′-GCA TGG CTC AGG AGT TTG TGA ACT GCA AGA TTC AG-3′, and as a reverse primer, 5′-CCT TTC ATA ACT GCA GAG CTC CAA TCT GCT TCA GC-3′. The 547-bp oligonucleotides for γ-GCS (rat γ-GCS sequence, BC081702) were obtained using 5′-CCT CTG GAG ACC AGA GTAT GGG AGT TAC-3′, and 5′-GCA GAT AGT GGC CAA CTG GTC ATA AAG G-3′. The 410-bp oligonucleotides for rat β-actin (BC063166) were obtained using, 5′-GAG CTA TGA GCT GCC TGA CG-3′, and 5′-AGC ATT TGC GGT GCA CGA TG-3′.

2.10. RNA interference and transfections

Double-stranded small interfering RNAs (siRNAs) corresponding to rat GRX1 DNA sequences (GenBank Accession No. NM-022278) (5′-ACU GCA AGA UUC AGU CUG GdTdT-3′ [siRNA-GRX-1] and 5′-AAC GUG GUC UCC UGG AAU UdTdT-3′ [siRNA-GRX-2]), and to rat LMW-PTP DNA sequences (NM-021262) (5′-CAC AUU GCA CGG CAG AUU AdTdT-3′ [siRNA-LMW-PTP-1] and 5′-UGA GAG AUC UGA AUA GAA AdTdT-3′ [siRNA-LMW-PTP-2]) were synthesized and annealed by Samchully Pharm Co., Ltd., Korea. siRNAs were transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with a final siRNA concentration of 100 nM.

2.11. Statistical analysis

Data were presented as the mean ± SD. Differences were examined by using ANOVA (StatView software). A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. DHEA suppresses PDGF-induced proliferation of A7r5 cells

The effect of DHEA on PDGF-induced cell proliferation was examined in A7r5 cells. Serum-starved A7r5 cells were cultured with or without 100 nM DHEA for 18 h, then in medium containing 0.2% FBS with or without 2 nM PDGF-BB. As shown in Fig. 1A, cell proliferation induced by PDGF-BB was suppressed by pretreatment with 100 nM DHEA. DHEA at 100 and 200 nM suppressed the PDGF-BB-induced proliferation in a dose-dependent manner (Fig. 1B). The data indicate that DHEA suppresses the PDGF-induced proliferation of A7r5 cells, consistent with the report by Williams et al. [12], in which the precise mechanism of the effect of DHEA on the PDGF-BB-induced proliferation of VSMC was not clear.

3.2. Suppression of VSMC marker genes by PDGF-BB is restored by DHEA

Next, the effect of DHEA on the expression of VSMC marker genes was examined. As VSMC marker genes, SMα-actin and SM22α were estimated by the quantitative RT-PCR method. After incubation with or without 100 nM DHEA for 18 h, A7r5 cells were treated with 2 nM PDGF-BB for 24 h. As shown in Fig. 1C, the expression of SM22α and SMα-actin was suppressed by treatment with PDGF-BB, whereas the suppressive effect of PDGF-BB on the VSMC phenotype was restored by DHEA. Taken together, DHEA attenuates the PDGF-induced proliferation and phenotypic switch in VSMC, suggesting that DHEA has some effects on the PDGF-BB-dependent cell signaling. Then, PDGF-BB-induced phosphorylation of PDGFR-β was examined.

3.3. DHEA down-regulates the PDGF-BB-dependent phosphorylation of PDGFR-β

PDGFR-β-mediated signals are particularly important for vascular remodeling and neointima formation [13]. As shown in Fig. 2A and B, A7r5 cells expressed abundant PDGFR-β. Then, phosphorylation of PDGFR-β was estimated by immunoblot analysis using rabbit antibodies against PDGFR-β and phosphor-(Tyr-751)-PDGFR-β. PDGF-BB-induced phosphorylation of PDGFR-β was observed at 5–30 min, with a peak at 5–10 min (lanes 2–3). Pretreatment with 100 nM DHEA suppressed the phosphorylation of PDGFR-β (lanes 6–7). The suppression of the phosphorylation of PDGFR-β by DHEA was dose-dependent (25–250 nM, data not shown). Then, the possible suppression of PDGFR-β-mediated signaling was examined.

3.4. DHEA induces the expression of GRX1 and γ-GCS mRNA and increases the level of GSH

PDGFR-mediated signaling is regulated by many factors. These factors are involved in the generation of reactive oxygen species and redox regulation [3]. We were interested in the role of the redox regulation of PDGFR-β by DHEA. The effect of DHEA on the mRNA expression of redox-related proteins, and the levels of GSH and oxidized glutathione (GSSG) were analyzed. A7r5 cells incubated with or without 100 nM DHEA for 18 h were treated with 2 nM PDGF-BB for 12 h. The expression was expressed as the relative intensity compared to the control. Treatment with DHEA increased GRX1 mRNA and γ-GCS, a rate-limiting enzyme for GSH synthesis (Fig. 2C). The level of GSH was increased by DHEA, while the level of GSSG was not changed by DHEA, resulting in the high GSH/GSSG ratio (Fig. 2D). These results indicate that DHEA increases the expression of GRX1 and γ-GCS to elevate the GSH/GRX1 redox potential as well as the GSH/GSSG ratio.

3.5. DHEA-dependent promoter activity of GRX1 and γ-GCS gene is regulated by PPARα

DHEA up-regulates the transcriptional activity mediated by PPARα [14,15]. This mechanism involves up-regulation of the expression of PPARα mRNA by DHEA [15]. To investigate the transcriptional regulation of GRX1 and γ-GCS by DHEA via the

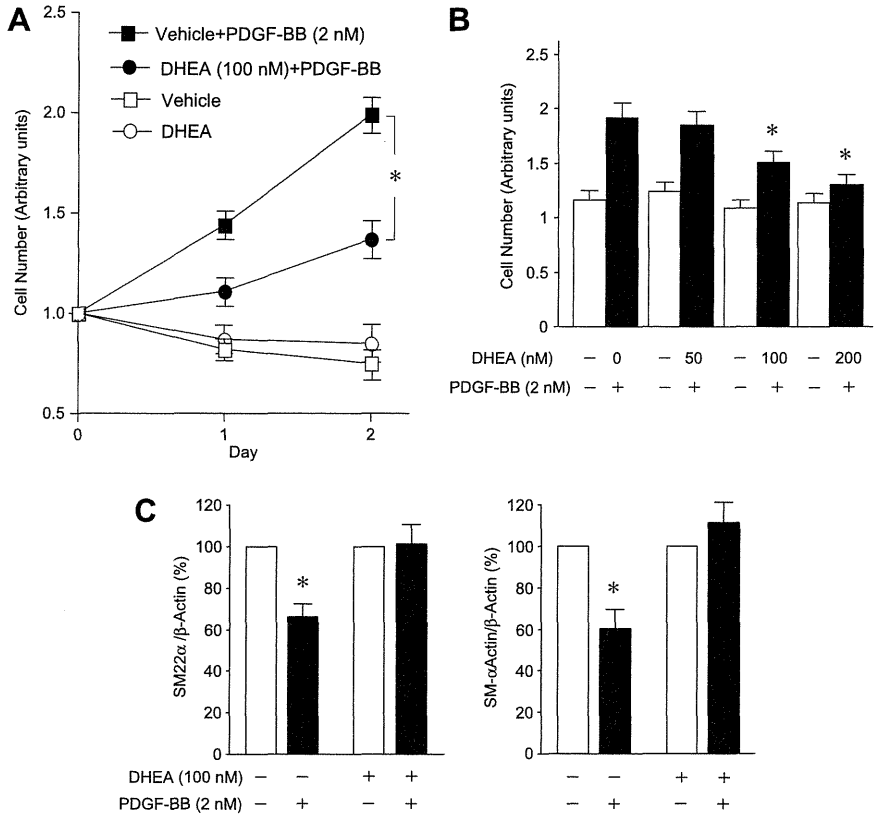

Fig. 1. Effect of DHEA on VSMC proliferation and expression of VSMC marker genes. Time-dependent (A) and dose-dependent (B) effects of DHEA on PDGF-BB-induced proliferation were estimated. (C) The expression of SM22α and SMα-actin was estimated by the quantitative RT-PCR method as described in Section 2. After incubation of A7r5 cells with or without 100 nM DHEA for 18 h, the cells were treated with 2 nM PDGF-BB for 24 h. Each value represents the mean for three independent experiments, and the SD was always within 10% of the mean. $^*p < 0.05$ compared with the control vehicle-treated cells.

PPARα-binding domain, a luciferase vector containing PPRE-like domain was constructed and introduced into A7r5 cells. The luciferase activity of the cells treated with DHEA for 18 h showed a 1.8-fold increase, but was almost lost when the PPRE-like site was deleted or mutated (Fig. 3A). Deletion of EpRE-like 2 or SP1 had no apparent effect on the DHEA-induced up-regulation of the luciferase activity (data not shown). Similarly, the promoter region of the γ-GCS heavy subunit containing a PPRE-like site was inserted into a luciferase vector. The luciferase activity of the cells treated with DHEA was stimulated by 1.6-fold, but was lost when the PPRE-like site was mutated (Fig. 3B). The results suggest that PPARα plays a role in the DHEA-induced up-regulation of the expression of GRX1 and γ-GCS.

3.6. DHEA maintained the redox state of LMW-PTP

PDGFR is a receptor-type tyrosine kinase, the activation of which is regulated by PDGF-dependent autophosphorylation and dephosphorylation by protein tyrosine phosphatases (PTPs). A number of tyrosine residues are phosphorylated in the cytosolic domain of PDGFR, leading to a site-specific recruitment of signal transduction molecules [4]. PTPs such as LMW-PTP are implicated in the control of PDGFR phosphorylation [16]. LMW-PTP is an 18-kDa enzyme that is widely expressed [17]. We focused on LMW-PTP because its redox-dependent regulation and the role in PDGF-BB/PDGFR signaling have been studied extensively [4]. Oxidative stress generated by PDGF-BB/PDGFR-β-mediated signaling causes the oxidization of LMW-PTP to form dithiothreitol-reducible high molecular weight oligomers, and the generation of peroxide is involved in the downregulation of LMW-PTP activity by PDGF-BB, leading to the activation of PDGFR-BB-dependent signaling pathways [3]. Fig. 4A shows that a decrease in the reduced form of LMW-PTP (the active form) was observed on treatment with PDGF-BB for 30 min (lane 2). The decrease in levels of the reduced form caused by PDGF-BB was similar to that observed on treatment with hydrogen peroxide (lane 5). Pretreatment of cells with DHEA for 18 h maintained the reduced form of LMW-PTP (lane 4). In this experiment, oligomerized LMW-PTP (the inactive form) was not detectable for unknown reason, and LMW-PTP-specific phosphatase activity was not determined. Together with our previous report using synthesized LMW-PTP and GSH/GRX1 redox system [3], the data suggest that DHEA maintained the redox state of LMW-PTP regulated by the GSH/GRX1 system.

3.7. Transfection of siRNAs for GRX1 and LMW-PTP abolishes the effect of DHEA on PDGF-BB-induced phosphorylation of PDGFR-β

To down-regulate the expression of GRX1 and LMW-PTP, specific siRNA (100 nM) were introduced into A7r5 cells. siRNAs bearing scrambled sequences were used as the control. At 48 h post-transfection, cells were serum-starved for 6 h, then stimulated with 2 nM PDGF-BB. Compared to the PDGF-BB-induced phosphorylation of PDGFR-β in the cells transfected with siRNA bearing scrambled sequences (Fig. 4B, lanes 2–4), the transfection of siRNA for GRX1 further stimulated the PDGF-BB-induced phosphorylation of PDGFR-β (lanes 5–8). Similarly, the cells transfected with the specific siRNA (100 nM) for LMW-PTP for 48 h showed an increase in PDGF-BB-induced phosphorylation of PDGFR-β (lanes 9–12). The results confirm that (i) DHEA increases the GSH/GRX1 redox potential, (ii) the GSH/GRX1 system is necessary to regulate the phosphorylation of PDGFR-β, (iii) the activity of LMW-PTP

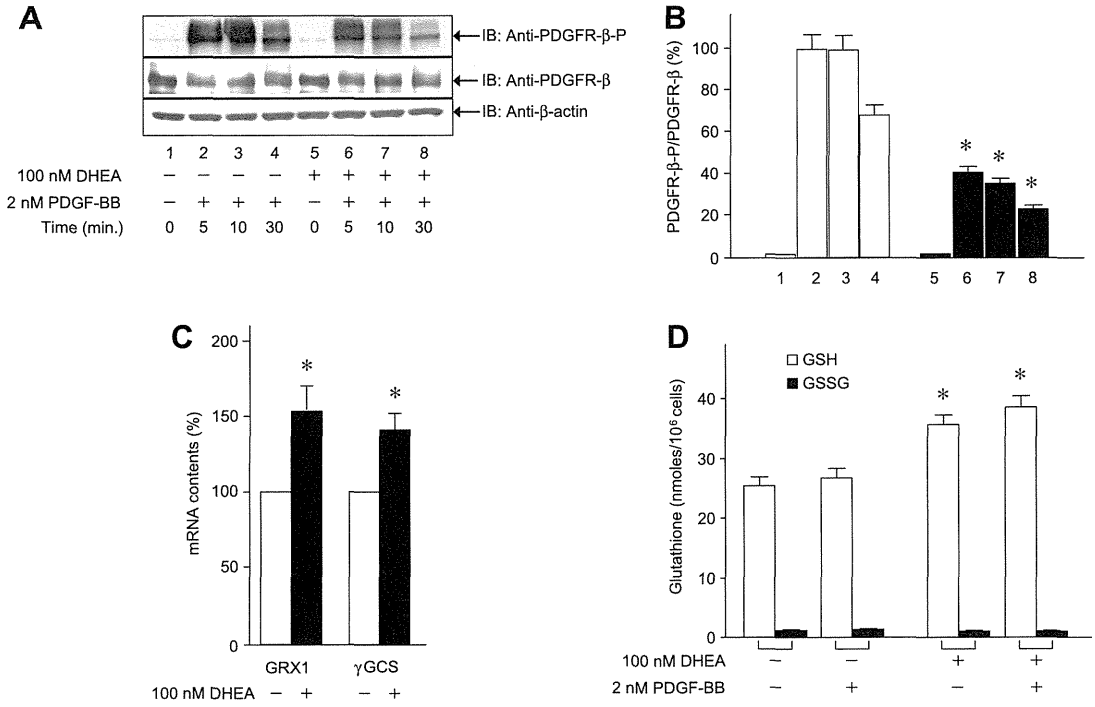

Fig. 2. DHEA down-regulates the PDGF-BB-dependent phosphorylation of PDGFR-β (A, B), and up-regulates the mRNA expression of GRX1 and γ-GCS (C) and GSH levels (D). A7r5 cells were serum-starved for 24 h. After pretreatment with 100 nM DHEA for 18 h, the cells were stimulated with 2 nM PDGF-BB for the periods indicated. (A) The phosphorylation status of PDGFR-β was examined by immunoblot analysis. (B) The intensity of the bands was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of phosphorylated PDGFR-β to PDGFR-β protein. (C) mRNA expression of GRX1 and γ-GCS heavy subunit was analyzed by quantitative RT-PCR. The expression was expressed as relative intensity compared to the control. (D) Levels of GSH and GSSG were estimated using a Total Glutathione Quantification Kit and were expressed as relative intensity compared to the control. Each value represents the mean for three independent experiments, and the SD was always within 10% of the mean. $^*p < 0.05$ compared with the control vehicle-treated cells.

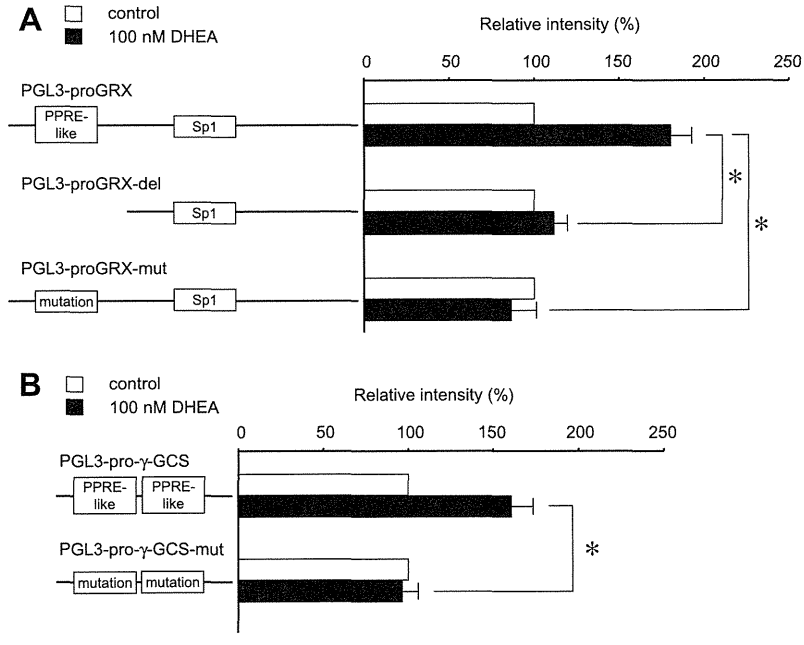

Fig. 3. PPRE-like element is important for the DHEA-dependent induction of the GRX1 and γ-GCS promoter in A7r5 cells. The transcriptional regulation of GRX1 and γ-GCS by DHEA was examined. The cells were transiently transfected with the GRX1 promoter-luciferase gene fusion plasmids (A) or the chimeric γ-GCS promoter-luciferase gene (B). After the transfection, luciferase activity was assayed with cellular extracts as described in Section 2.

maintained by DHEA regulates the phosphorylation, of PDGFR-β and (iv) the LMW-PTP-dependent dephosphorylation is regulated by the GSH/GRX1 system. To know whether the DHEA-dependent suppression of the PDGF-BB-induced phosphorylation of PDGFR-β is mediated through specific receptors for androgen or estradiol, the effect of antagonists against androgen receptor (flutamide) and estrogen receptor α/β (ICI182,780) was examined. It was found that the antagonists had no apparent effect on the DHEA-induced

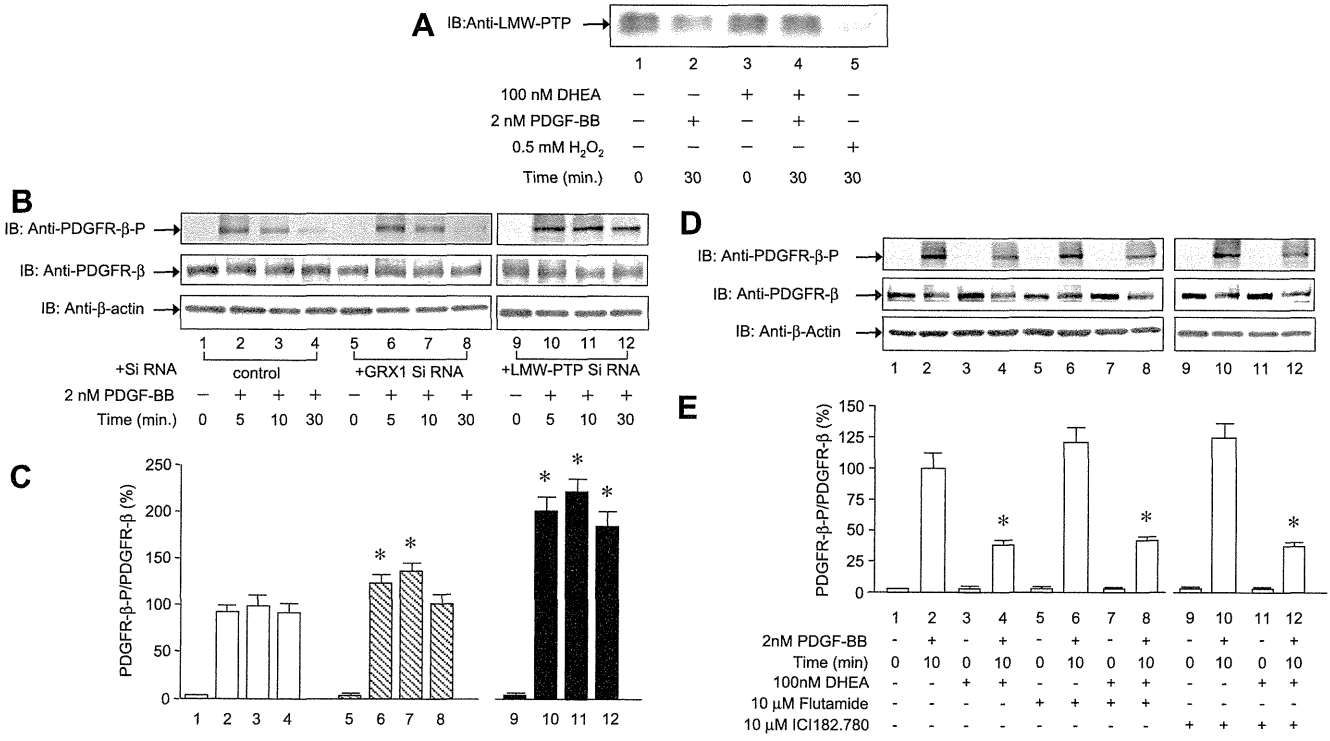

Fig. 4. The role of LMW-PTP, GRX1 and sex hormone receptors in the effects of DHEA on PDGF-BB-induced phosphorylation of PDGFR-β. (A) Effect of DHEA on the redox state of LMW-PTP was estimated. The redox state of proteins was assessed by modifying free thiol with AMS as described in Section 2. (B, C) To down-regulate the expression of GRX1, a specific siRNA for GRX1 (100 nM) was introduced into A7r5 cells. siRNA bearing a scrambled sequence was used as the control. Similarly, specific siRNA for LMW-PTP (100 nM) was introduced into the cells. (D) Effects of an androgen receptor antagonist (flutamide) and an estrogen receptor α/β antagonist (ICI182,780) on DHEA-induced suppression of PDGF-BB-dependent phosphorylation of PDGFR-β were examined. (E) The intensity of the bands was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of phosphorylated PDGFR-β to PDGFR-β protein.

suppression of the phosphorylation (Fig. 4D, lane 4 vs. lane 8 and lane 12). The results are consistent with the previous report that the inhibitory effect of DHEA on PDGF-BB-induced proliferation of VSMC is independent of the androgen receptor and estrogen receptor [12].

In summary, this study showed that DHEA inhibits PDGFR-β phosphorylation that leads to proliferation and phenotypic changes of VSMC, and that the transcriptional control of the GSH/GRX level and the redox state of LMW-PTP may account, at least in part, for the beneficial effects of DHEA on VSMC.

Acknowledgments

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ORIGINAL ARTICLE

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

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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

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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.

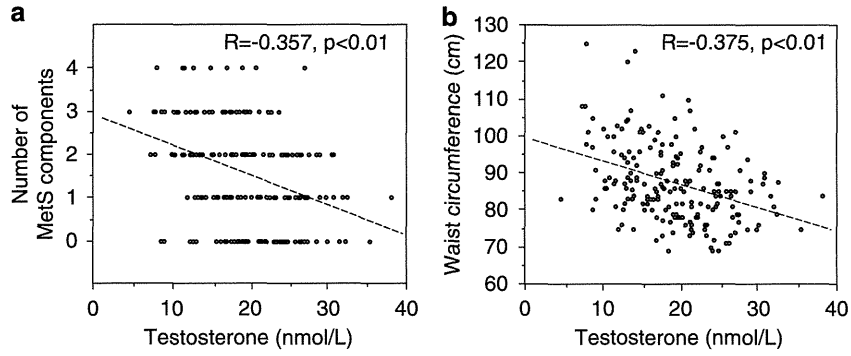

Figure 1 Scattergrams and regression lines (dotted lines) showing the correlation between testosterone and the number of metabolic syndrome (MetS) components (**a**) or waist circumference (**b**).

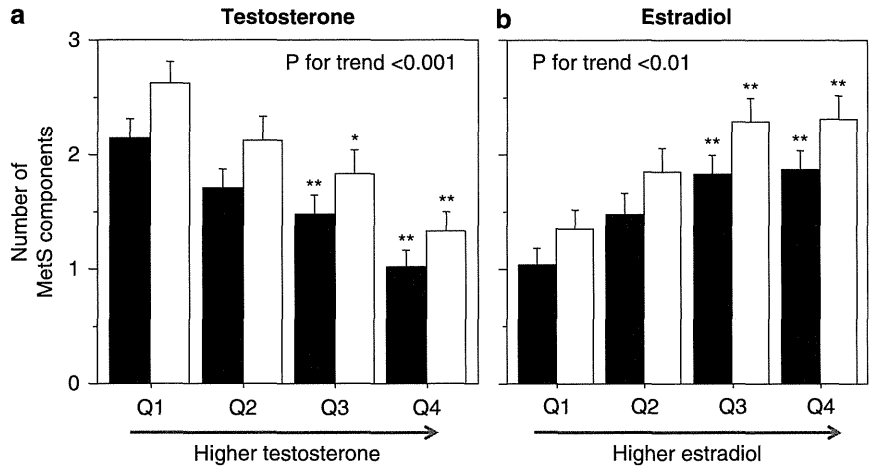

Figure 2 Number of metabolic syndrome (MetS) components according to quartiles of plasma testosterone (**a**) and estradiol (**b**). MetS components were defined according to the Japanese criteria (closed bars) and the IDF criteria for Japanese ethnicity (open bars). Values are expressed as the mean ± 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

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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-

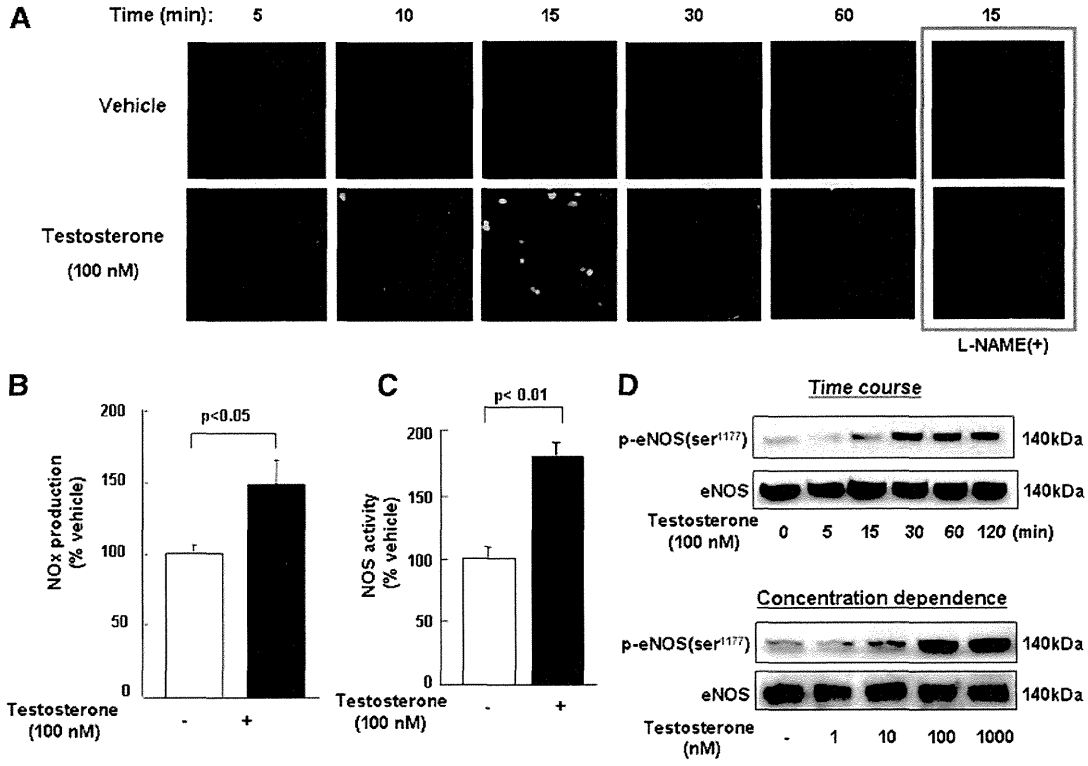

FIG. 1. Testosterone rapidly stimulates NO production and NOS activation in HAEC. A, Growth-arrested HAEC were loaded with DAF-2DA before treatment with or without testosterone for the indicated times. After testosterone treatment, cells were fixed in 2% paraformaldehyde and viewed using a fluorescence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

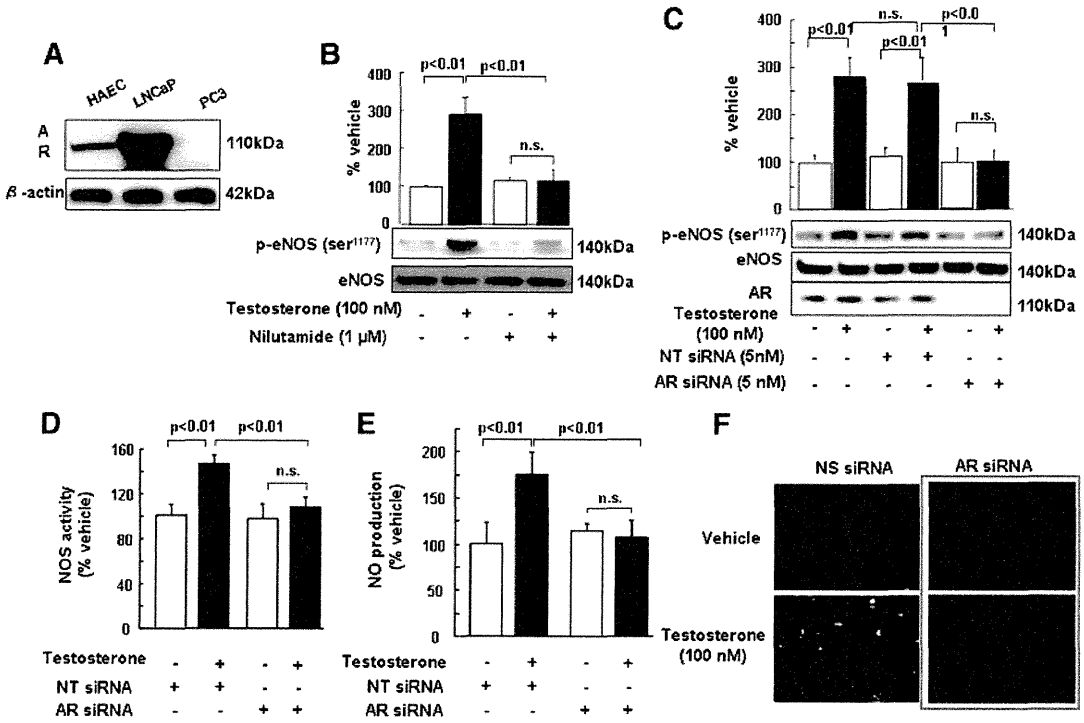

FIG. 2. AR is involved in testosterone-induced eNOS phosphorylation. A, AR expression was examined in HAEC, AR-positive LNCaP cells, and AR-negative PC3 cells using immunoblotting (*upper panel*). The membrane was stripped and re-immunoblotted with anti-β-actin antibody for monitoring equal amounts of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

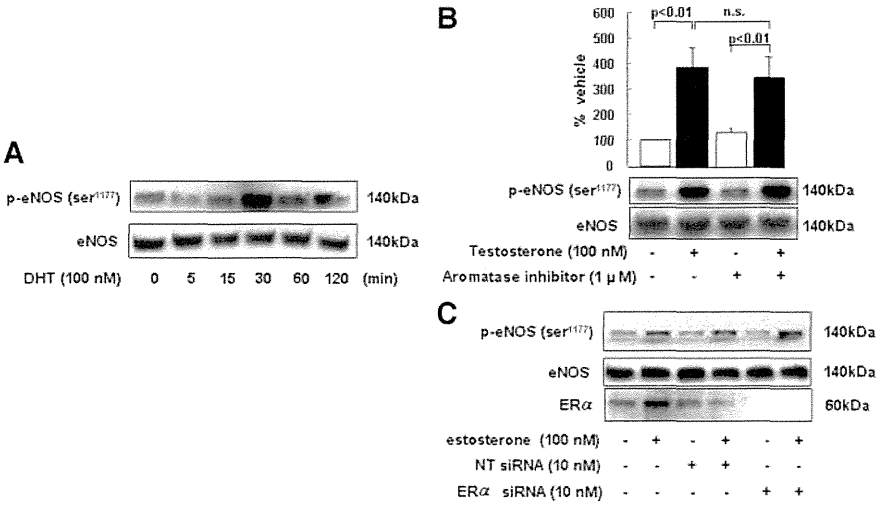

FIG. 3. Estradiol/ER pathway is not involved in testosterone-induced eNOS phosphorylation. A, Growth-arrested HAEC were treated with DHT or vehicle for the indicated times. B, Growth-arrested cells were treated with testosterone or vehicle for 30 min in the presence or absence of aromatase inhibitor.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

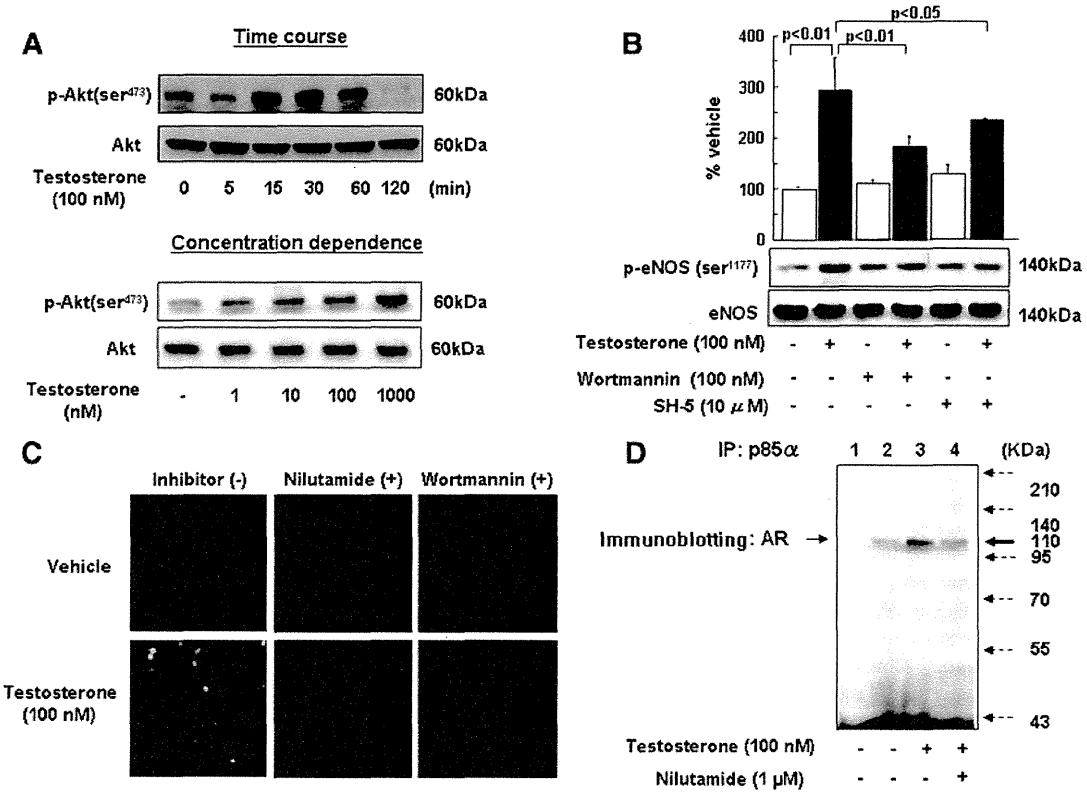

FIG. 4. PI3-kinase/Akt pathway is involved in testosterone-induced eNOS phosphorylation and NO production. A, Growth-arrested HAEC were treated with vehicle or testosterone for the indicated times or with the indicated concentrations of testosterone for 30 min. Phosphorylation of Akt at Ser473 (p-Akt) 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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