THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 10, pp. 7537–7544, March 5, 2010

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

Received for publication, August 17, 2009, and in revised form, December 16, 2009 Published, JBC Papers in Press, January 4, 2010, DOI 10.1074/jbc.M109.055087

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.

* This work was supported by Health and Labor Sciences Research Grant H17-Choju-046 from the Ministry of Health, Labor, and Welfare of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, and Sports of Japan 21390220 and 20249041.

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

[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.

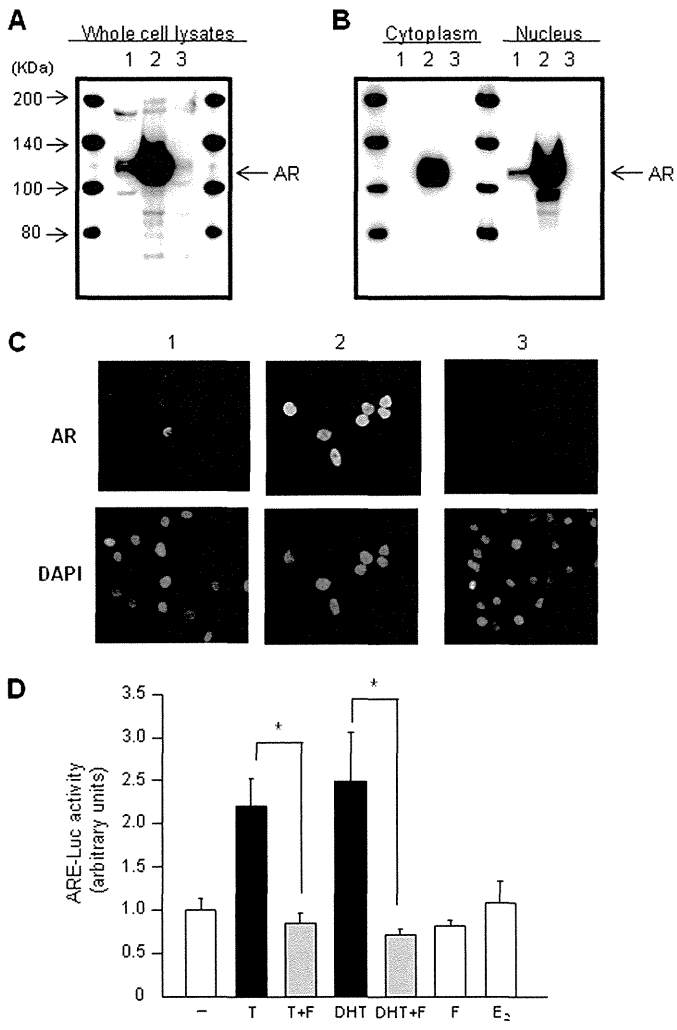


FIGURE 1. **Expression of the functional the AR in HASMC.** Endogenous expression of the AR in HASMC (*lane 1*) was examined in whole cell lysates (*A*) and cytoplasmic and nuclear fractions (*B*) compared with that in human prostate cancer cell lines, LNCaP (AR-positive; *lane 2*) and PC-3 (AR-negative;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

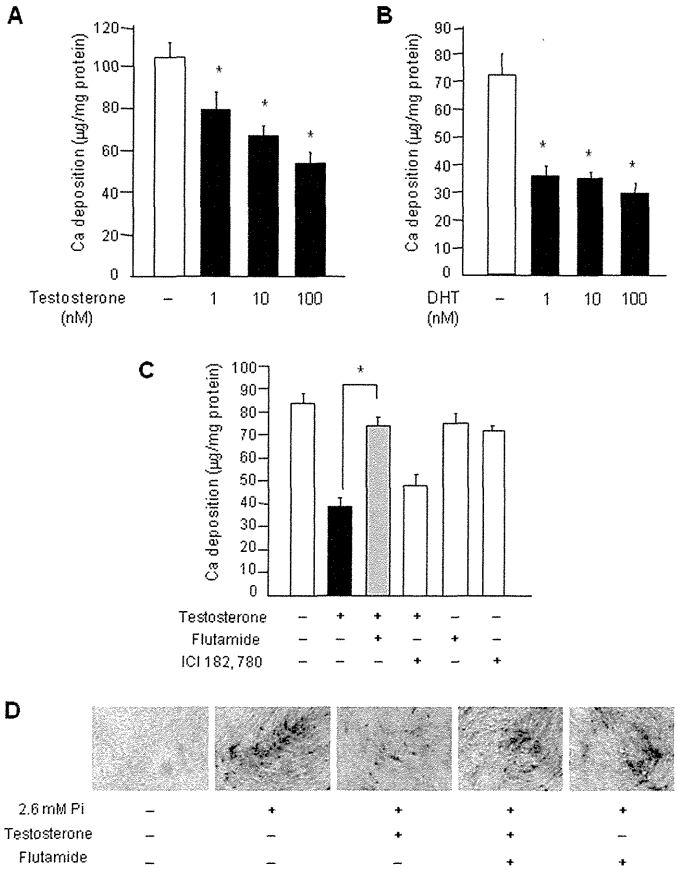


FIGURE 2. **Androgens prevent P_i-induced calcification via the AR.** HASMC were cultured with the indicated concentrations of androgens (testosterone (*A*) and DHT (*B*)) in the presence of 2.6 mM P_i for 6 days. Calcium deposition was measured by the *o*-cresolphthalein complexone method and normalized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

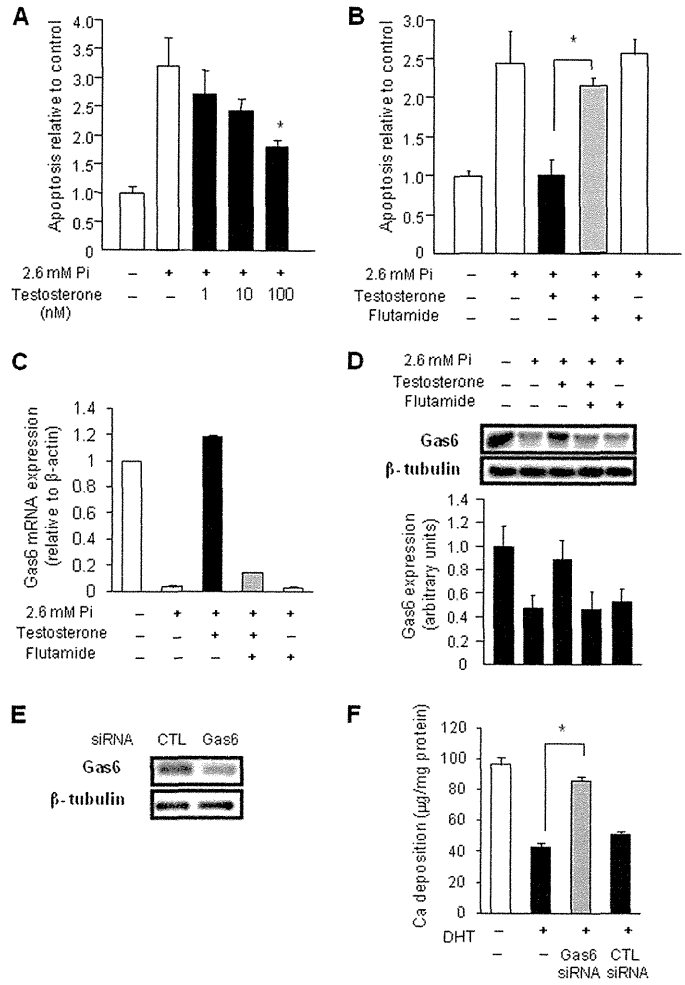


FIGURE 3. **Androgens inhibit P_i-induced apoptosis and restore Gas6-mediated survival pathway.** *A*, HASMC were cultured with the indicated concentrations of testosterone in the presence of 2.6 mM P_i for 6 days. A quantitative index of apoptosis, determined by DNA fragmentation enzyme-linked immunosorbent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

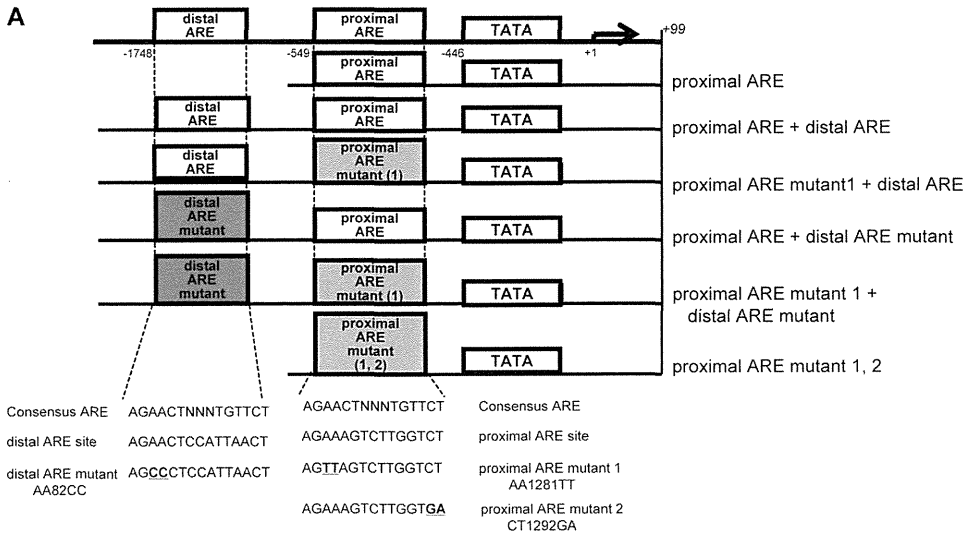
Because apoptosis is a crucial and initiating event in P_i-induced VSMC calcification (16, 17), we examined whether androgens inhibit P_i-induced apoptosis. Furthermore, in our recent study apoptosis induced by P_i has been shown to be associated with inhibition of Gas6 expression and secretion (16,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

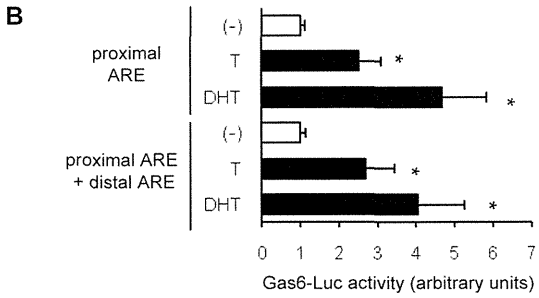
The preventive effect of Gas6 on P_i-induced apoptosis and calcification is mediated by the phosphatidylinositol 3-OH kinase/Akt pathway, a well known anti-apoptotic signaling pathway, through Bcl2 family proteins (17). We found that testosterone restored the Akt phosphorylation down-regulated by P_i, 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*).

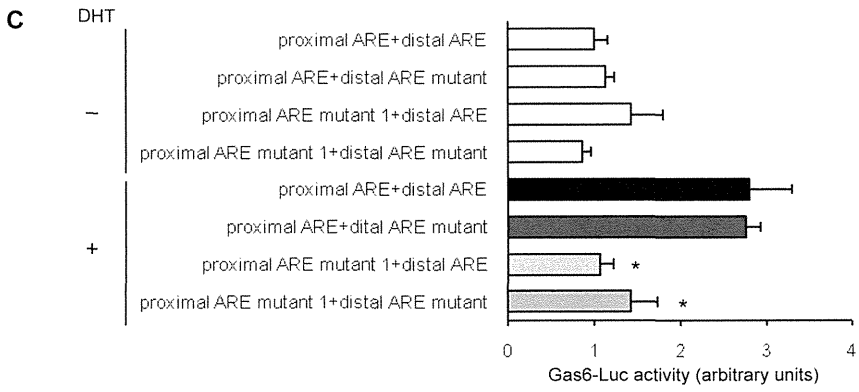
To determine whether Gas6 is required for androgen-mediated effects, we blocked the action of Gas6 using siRNA (Fig. 3*E*) and examined the effect of androgens on P_i-induced calcification. As shown in Fig. 3*F*, knockdown of the Gas6 gene significantly reversed the inhibitory effect of androgens on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

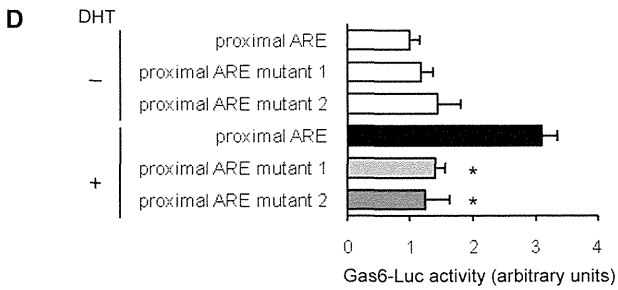
The Proximal ARE in Gas6 Promoter Is Essential for Androgen-stimulated Gas6 Transcriptional Activation—To investigate the molecular mechanism involved in up-regulation of Gas6 expression by androgens, we explored the existence of ARE sites in the promoter region of the Gas6 gene (−1827 to +99 bp). We 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









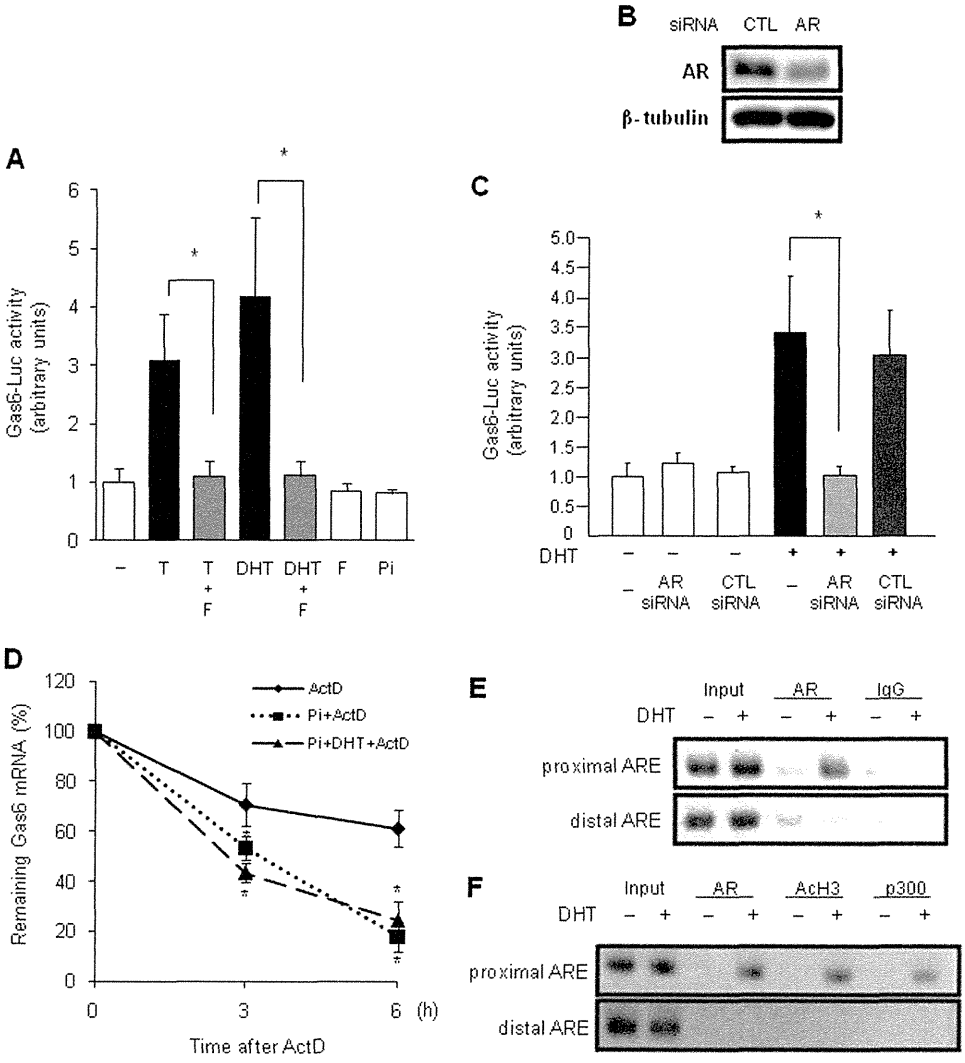


FIGURE 5. **Interaction of the AR with the proximal ARE is essential for transactivation of Gas6 gene by androgen.** *A*, HASMC were transfected with the Gas6-luc construct containing the proximal ARE. Twenty-four hours after transfection, testosterone (*T*, 100 nM), DHT (100 nM), P_i (*Pi*, 2.6 mM),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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

Acknowledgments—We thank Yuki Ito for technical assistance and Prof. Satoshi Inoue, Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, for providing the LNCaP and PC3 cells.

REFERENCES

1. Simon, D., Charles, M. A., Nahoul, K., Orssaud, G., Kremski, J., Hully, V., Joubert, E., Papoz, L., and Eschwege, E. (1997) *J. Clin. Endocrinol. Metab.* **82,** 682–685
2. Fogari, R., Preti, P., Zoppi, A., Fogari, E., Rinaldi, A., Corradi, L., and Mugellini, A. (2005) *Hypertens. Res.* **28,** 625–630
3. Stellato, R. K., Feldman, H. A., Hamdy, O., Horton, E. S., and McKinlay, J. B. (2000) *Diabetes Care* **23,** 490–494
4. Shores, M. M., Matsumoto, A. M., Sloan, K. L., and Kivlahan, D. R. (2006) *Arch. Intern. Med.* **166,** 1660–1665
5. Jeppesen, L. L., Jørgensen, H. S., Nakayama, H., Raaschou, H. O., Olsen, T. S., and Winther, K. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16,** 749–754
6. Bruck, B., Brehme, U., Gugel, N., Hanke, S., Finking, G., Lutz, C., Benda, N., Schmahl, F. W., Haasis, R., and Hanke, H. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 2192–2199
7. Tharp, D. L., Masseau, I., Ivey, J., Ganjam, V. K., and Bowles, D. K. (2009) *Cardiovasc. Res.* **82,** 152–160
8. Nathan, L., Shi, W., Dinh, H., Mukherjee, T. K., Wang, X., Lusis, A. J., and Chaudhuri, G. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 3589–3593
9. Hak, A. E., Witteman, J. C., de Jong, F. H., Geerlings, M. I., Hofman, A., and Pols, H. A. (2002) *J. Clin. Endocinol. Metab.* **87,** 3632–3639
10. Mooradian, A. D., Morley, J. E., and Korenman, S. G. (1987) *Endocr. Rev.* **8,** 1–28
11. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83,** 835–839
12. Heinlein, C. A., and Chang, C. (2004) *Endocr. Rev.* **25,** 276–308
13. Liu, P. Y., Christian, R. C., Ruan, M., Miller, V. M., and Fitzpatrick, L. A. (2005) *J. Clin. Endocrinol. Metab.* **90,** 1041–1046
14. Hanke, H., Lenz, C., Hess, B., Spindler, K. D., and Weidemann, W. (2001) *Circulation* **103,** 1382–1385
15. Zitzmann, M., Brune, M., Kornmann, B., Gromoll, J., von Eckardstein, S., von Eckardstein, A., and Nieschlag, E. (2001) *J. Clin. Endocrinol. Metab.* **86,** 4867–4873
16. Son, B. K., Kozaki, K., Iijima, K., Eto, M., Kojima, T., Ota, H., Senda, Y., Maemura, K., Nakano, T., Akishita, M., and Ouchi, Y. (2006) *Circ. Res.* **98,** 1024–1031
17. Son, B. K., Kozaki, K., Iijima, K., Eto, M., Nakano, T., Akishita, M., and Ouchi, Y. (2007) *Eur. J. Pharmacol.* **556,** 1–8
18. Mark, M. R., Chen, J., Hammonds, R. G., Sadick, M., and Godowsk, P. J. (1996) *J. Biol. Chem.* **271,** 9785–9789
19. Zhao, Y., Takeyama, K., Sawatsubashi, S., Ito, S., Suzuki, E., Yamagata, K., Tanabe, M., Kimura, S., Fujiyama, S., Ueda, T., Murata, T., Matsukawa, H.,

Shirode, Y., Kouzmenko, A. P., Li, F., Tabata, T., and Kato, S. (2009) *Mol. Cell. Biol.* **29,** 1017–1034
20. Son, B. K., Akishita, M., Iijima, K., Kozaki, K., Maemura, K., Eto, M., and Ouchi, Y. (2008) *Endocrinology* **149,** 1646–1653
21. Yaron, M., Greenman, Y., Rosenfeld, J. B., Izkhakov, E., Limor, R., Osher, E., Shenkerman, G., Tordjman, K., and Stern, N. (2009) *Eur. J. Endocrinol.* **160,** 839–846
22. Alexandersen, P., Haarbo, J., Byrjalsen, I., Lawaetz, H., and Christiansen, C. (1999) *Circ. Res.* **84,** 813–819
23. Ong, P. J., Patrizi, G., Chong, W. C., Webb, C. M., Hayward, C. S., and Collins, P. (2000) *Am. J. Cardiol.* **85,** 269–272
24. Rosano, G. M., Leonardo, F., Pagnotta, P., Pelliccia, F., Panina, G., Cerquetani, E., della Monica, P. L., Bonfigli, B., Volpe, M., and Chierchia, S. L. (1999) *Circulation* **99,** 1666–1670
25. Emi, Y., Adachi, M., Sasaki, A., Nakamura, Y., and Nakatsuka, M. (2008) *J. Obstet. Gynaecol. Res.* **34,** 890–897
26. Mäkinen, J., Järvisalo, M. J., Pöllänen, P., Perheentupa, A., Irjala, K., Koskenvuo, M., Mäkinen, J., Huhtaniemi, I., and Raitakari, O. T. (2005) *J. Am. Coll. Cardiol.* **45,** 1603–1608
27. Akishita, M., Hashimoto, M., Ohike, Y., Ogawa, S., Iijima, K., Eto, M., and Ouchi, Y. (2007) *Hypertens. Res.* **30,** 1029–1034
28. van den Beld, A. W., Bots, M. L., Janssen, J. A., Pols, H. A., Lamberts, S. W., and Grobbee, D. E. (2003) *Am. J. Epidemiol.* **157,** 25–31
29. Khaw, K. T., Dowsett, M., Folkerd, E., Bingham, S., Wareham, N., Luben, R., and Welch, A., Day, N. (2007) *Circulation* **116,** 2694–2701
30. Laughlin, G. A., Barrett-Connor, E., and Bergstrom, J. (2008) *J. Clin. Endocrinol. Metab.* **93,** 68–75
31. McRobb, L., Handelsman, D. J., and Heather, A. K. (2009) *Endocrinology* **150,** 841–848
32. Heinlein, C. A., and Chang, C. (2002) *Endocr. Rev.* **23,** 175–200
33. Ikeda, Y., Aihara, K., Yoshida, S., Sato, T., Yagi, S., Iwase, T., Sumitomo, Y., Ise, T., Ishikawa, K., Azuma, H., Akaike, M., Kato, S., and Matsumoto, T. (2009) *Endocrinology* **150,** 2857–2864
34. Read, J. T., Rahmani, M., Boroomand, S., Allahverdian, S., McManus, B. M., and Rennie, P. S. (2007) *J. Biol. Chem.* **282,** 31954–31963
35. Heemers, H., Verrijdt, G., Organe, S., Claessens, F., Heyns, W., Verhoeven, G., and Swinnen, J. V. (2004) *J. Biol. Chem.* **279,** 30880–30887
36. Mo, R., Tony, Zhu, Y., Zhang, Z., Rao, S. M., and Zhu, Y. J. (2007) *Biochem. Biophys. Res. Commun.* **353,** 189–194
37. Nakao, J., Orimo, H., Ooyama, T., and Shiraki, M. (1979) *Atherosclerosis* **34,** 469–474
38. Manson, J. E., Allison, M. A., Rossouw, J. E., Carr, J. J., Langer, R. D., Hsia, J., Kuller, L. H., Cochrane, B. B., Hunt, J. R., Ludlam, S. E., Pettinger, M. B., Gass, M., Margolis, K. L., Nathan, L., Ockene, J. K., Prentice, R. L., Robbins, J., and Stefanick, M. L., (2007) *N. Engl. J. Med.* **356,** 2591–2602
39. Alexandersen, P., Tankó, L. B., Bagger, Y. Z., Qin, G., and Christiansen, C. (2006) *Climacteric* **9,** 108–118
40. Rzewuska-Lech, E., Jayachandran, M., Fitzpatrick, L. A., and Miller, V. M. (2005) *Am. J. Physiol. Endocrinol. Metab.* **289,** E105–E112
41. Nakamura, T., Akishita, M., Kozaki, K., Toba, K., Orimo, H., and Ouchi, Y. (2003) *Geriatr. Gerontol. Int.* **3,** 145–149
42. Balica, M., Boström, K., Shin, V., Tillisch, K., and Demer, L. L. (1997) *Circulation* **95,** 1954–1960

Atherosclerosis 210 (2010) 232–236

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Low testosterone level as a predictor of cardiovascular events in Japanese men with coronary risk factors

Masahiro Akishita[a,*], Masayoshi Hashimoto[b], Yumiko Ohike[a], Sumito Ogawa[a], Katsuya Iijima[a], Masato Eto[a], Yasuyoshi Ouchi[a]

[a] Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[b] Department of General Internal Medicine, Kobe University School of Medicine, Kobe, Japan

ARTICLE INFO

Article history:
Received 25 August 2009
Received in revised form 5 October 2009
Accepted 22 October 2009
Available online 13 November 2009

Keywords:
Androgen
Sex hormone
Estrogen
Risk factor

ABSTRACT

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

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

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

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

1. Introduction

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

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

* Corresponding author. Tel.: +81 3 5800 8832; fax: +81 3 5800 8831.
E-mail address: akishita-tky@umin.ac.jp (M. Akishita).

0021-9150/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2009.10.037

2. Methods

2.1. Subjects

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

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

2.2. Clinical measurements

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

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

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

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

2.3. Follow-up

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

2.4. Data analysis

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

3. Results

3.1. Characteristics of subjects according to plasma testosterone level

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

3.2. CV events and hormones

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

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

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

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

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

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

Table 2
Cardiovascular events by tertile of plasma testosterone.

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

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

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

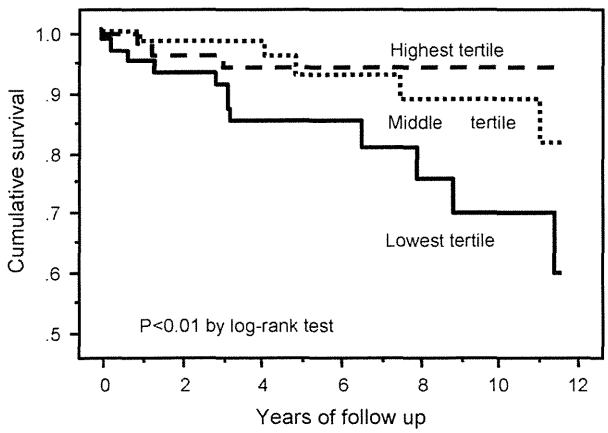


Fig. 1. Survival curves for cardiovascular events by tertile group of plasma concentration of testosterone. Cut-offs of the tertiles for testosterone were 14.2 and 19.4 nmol/L (410 and 560 ng/dL).

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

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

4. Discussion

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

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

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

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

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

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

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

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

Acknowledgements

We thank Ms. Yuki Ito for her excellent technical assistance. This study was supported by a Health and Labor Sciences Research Grant (H17-Choju-046) from the Ministry of Health, Labour and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (21390220, 20249041) and grants from the NOVARTIS Foundation for Gerontological Research and the Yamaguchi Endocrine Research Association.

References

[1] Muller M, den Tonkelaar I, Thijssen JH, Grobbee DE, van der Schouw YT. Endogenous sex hormones in men aged 40–80 years. Eur J Endocrinol 2003;149:583–9.
[2] Kaufman JM, Vermeulen A. The decline of androgen levels in elderly men and its clinical and therapeutic implications. Endocr Rev 2005;26:833–76.
[3] Fukai S, Akishita M, Yamada S, et al. Association of plasma sex hormone levels with functional decline in elderly men and women. Geriatr Gerontol Int 2009;9:282–9.
[4] Hak AE, Witteman JC, de Jong FH, et al. Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men: the Rotterdam study. J Clin Endocrinol Metab 2002;87:3632–9.
[5] Simon D, Charles MA, Nahoul K, et al. Association between plasma total testosterone and cardiovascular risk factors in healthy adult men: The Telecom Study. J Clin Endocrinol Metab 1997;82:682–5.
[6] Fogari R, Preti P, Zoppi A, et al. Serum testosterone levels and arterial blood pressure in the elderly. Hypertens Res 2005;28:625–30.
[7] Stellato RK, Feldman HA, Hamdy O, Horton ES, McKinlay JB. Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. Diabetes Care 2000;23:490–4.
[8] Akishita M, Hashimoto M, Ohike Y, et al. Low testosterone level is an independent determinant of endothelial dysfunction in men. Hypertens Res 2007;30:1029–34.
[9] van den Beld AW, Bots ML, Janssen JA, et al. Endogenous hormones and carotid atherosclerosis in elderly men. Am J Epidemiol 2003;157:25–31.
[10] Shores MM, Matsumoto AM, Sloan KL, Kivlahan DR. Low serum testosterone and mortality in male veterans. Arch Intern Med 2006;166:1660–5.

[11] Khaw KT, Dowsett M, Folkerd E, et al. Endogenous testosterone and mortality due to all causes, cardiovascular disease, and cancer in men: European prospective investigation into cancer in Norfolk (EPIC-Norfolk) Prospective Population Study. Circulation 2007;116:2694–701.
[12] Laughlin GA, Barrett-Connor E, Bergstrom J. Low serum testosterone and mortality in older men. J Clin Endocrinol Metab 2008;93:68–75.
[13] Ogihara T, Kikuchi K, Matsuoka H, et al. The Japanese Society of Hypertension guidelines for the management of hypertension (JSH 2009). Hypertens Res 2009;32:3–107.
[14] Teramoto T, Sasaki J, Ueshima H, et al. Japan Atherosclerosis Society (JAS) Committee for epidemiology and clinical management of atherosclerosis. Diagnostic criteria for dyslipidemia. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese. J Atheroscler Thromb 2007;14:155–8.
[15] Kuzuya T, Nakagawa S, Satoh J, et al. Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. Diabetes Res Clin Pract 2002;55:65–85.
[16] Kobayashi K, Akishita M, Yu W, et al. Interrelationship between non-invasive measurements of atherosclerosis; flow-mediated dilation of brachial artery, carotid intima-media thickness and pulse wave velocity. Atherosclerosis 2004;173:13–8.
[17] Feldman HA, Johannes CB, McKinlay JB, Longcope C. Low dehydroepiandrosterone sulfate and heart disease in middle-aged men: cross-sectional results from the Massachusetts Male Aging Study. Ann Epidemiol 1998;8:217–28.
[18] Jeppesen LL, Jorgensen HS, Nakayama H, et al. Decreased serum testosterone in men with acute ischemic stroke. Arterioscler Thromb Vasc Biol 1996;16:749–54.
[19] Araujo AB, Kupelian V, Page ST, et al. Sex steroids and all-cause and cause-specific mortality in men. Arch Intern Med 2007;167:1252–60.
[20] Smith GD, Ben-Shlomo Y, Beswick A, et al. Cortisol, testosterone, and coronary heart disease: prospective evidence from the Caerphilly study. Circulation 2005;112:332–40.
[21] Feldman HA, Johannes CB, Araujo AB, et al. Low dehydroepiandrosterone and ischemic heart disease in middle-aged men: prospective results from the Massachusetts Male Aging Study. Am J Epidemiol 2001;153:79–89.
[22] Arnlov J, Pencina MJ, Amin S, et al. Endogenous sex hormones and cardiovascular disease incidence in men. Ann Intern Med 2006;145:176–84.
[23] Emmelot-Vonk MH, Verhaar HJ, Nakhai Pour HR, et al. Effect of testosterone supplementation on functional mobility, cognition, and other parameters in older men: a randomized controlled trial. JAMA 2008;299:39–52.
[24] Ong PJ, Patrizi G, Chong WC, et al. Testosterone enhances flow-mediated brachial artery reactivity in men with coronary artery disease. Am J Cardiol 2000;85:269–72.
[25] Tharp DL, Masseau I, Ivey J, Ganjam VK, Bowles DK. Endogenous testosterone attenuates neointima formation after moderate coronary balloon injury in male swine. Cardiovasc Res 2009;82:152–60.
[26] Jones TH, Saad F. The effects of testosterone on risk factors for, and the mediators of, the atherosclerotic process. Atherosclerosis 2009 April 24 [Epub ahead of print].
[27] Fogari R, Preti P, Derosa G, et al. Effect of antihypertensive treatment with valsartan or atenolol on sexual activity and plasma testosterone in hypertensive men. Eur J Clin Pharmacol 2002;58:177–80.

Geriatrics Gerontology INTERNATIONAL 1st Impact Factor in June 2010 Medline Indexed

Geriatr Gerontol Int 2010; **10 (Suppl. 1):** *S137–S147*

Muscle weakness and neuromuscular junctions in aging and disease

Kazuhiro Shigemoto,[1] Sachiho Kubo,[1] Syuuichi Mori,[1] Shigeru Yamada,[1] Takuyu Akiyoshi[1,2] and Tsuyoshi Miyazaki[1]

[1]*Research Team for Molecular Biomarkers, Tokyo Metropolitan Institute of Gerontology, and* [2]*Department of Geriatric Medicine, Tokyo University School of Medicine, Itabashi, Tokyo, Japan*

A critical issue in today's super-aging society is the need to reduce the burden of family care while continuing to make our medical institutions supportive. A rapidly emerging, major health concern is the debilitating effect of muscle weakness and atrophy from aging, termed sarcopenia; however, the molecular basis of this condition is not well understood. Our research aim is to elucidate the molecular mechanisms of age-related muscle atrophy and to devise new measures for preventing and treating this disability. A promising treatment for muscle atrophy is the promotion of muscle regeneration by recruiting stem cells into the targeted region. The first requirement is to understand how the motor system, which consists of muscles and motoneurons, is maintained to accomplish that goal. Recent studies in the field of neuroscience have focused on neuromuscular junctions (NMJ), which play important roles in the maintenance of both motor nerves and muscle fibers. Signaling between muscles and motoneurons at NMJ supports interactions within the motor system. To understand the mechanisms involved, we focus our research on the pathogenic processes underlying neuromuscular diseases. The well-known autoimmune disease, myasthenia gravis (MG), serves as a model not only for tracking the pathogenesis and treatment outcomes of all autoimmune diseases, but also for understanding synaptic functions in maintaining the motor system. Here, we describe recent insights into the molecular mechanisms required for the maintenance of NMJ and the related causes of muscle atrophy. **Geriatr Gerontol Int 2010; 10 (Suppl. 1): S137–S147.**

Keywords: muscle-specific kinase, myasthenia gravis, neuromuscular junction, sarcopenia.

Neuromuscular junctions

Neuromuscular junctions (NMJ), which are structures located between motor terminals and muscles, are the sites of synapses between motor nerves and muscle fibers. At the anterior horn of the spinal cord and brainstem, skeletal muscle fibers are innervated by large motor neurons. The terminal arborization of each α-motor neuron is situated in a shallow depression of the muscle cell membrane, which is invaginated further into deep and regular folds, termed postjunctional folds (Fig. 1). The motor nerve terminal is specialized for neurotransmitter (acetylcholine; ACh) release. Synaptic vesicles containing ACh cluster adjacent to specialized structures of the presynaptic membrane, called active zones. The active zones are aligned precisely with mouths of the post-junctional folds. ACh receptors (AChR) are highly concentrated, with a density of about 12 000 receptors per μm^2, at the post-junctional membrane nearest to the fold's peak (Fig. 1). When the nerve action potential reaches the terminal, depolarization opens voltage-gated Ca^+ channels on the presynaptic membrane. This allows a Ca^+ influx that triggers the

Accepted for publication 20 November 2009.

Correspondence: Dr Kazuhiro Shigemoto MD PhD, Tokyo Metropolitan Institute of Gerontology, Research Team for Molecular Biomarkers, Sakaecho 35-2, Itabashi-ku, Tokyo 173-0015, Japan. Email: kazshige@tmig.or.jp

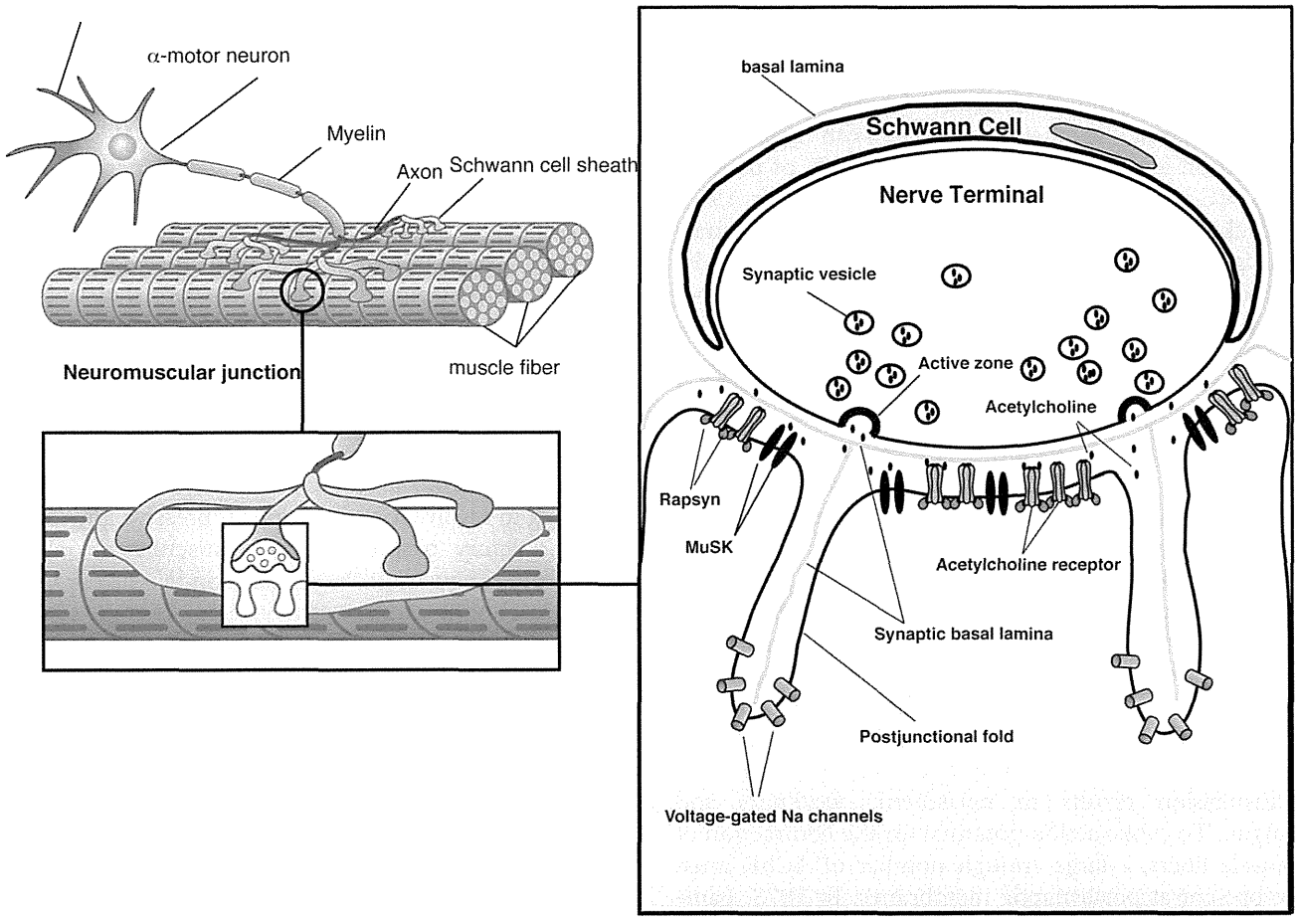


Figure 1 Structure and molecular architecture of the neuromuscular junctions (NMJ). Drawings show progressive enlargement of segments of a NMJ. The presynaptic terminals consist of multiple swellings called synaptic terminals covered by a thin layer of Schwann cells. The nerve terminal occupies a shallow gutter in the muscle fiber and is capped by processes of Schwann cells. Acetylcholine (ACh) from 150 to 200 vesicles is released from the active zones in the nerve terminal, which directly oppose junctional folds in the postsynaptic membrane. The terminals are separated from the postsynaptic cell by the synaptic cleft, which is about 50 nm wide. Acetylcholine receptors, muscle-specific kinase (MuSK) and rapsyn concentrated at the peaks of postsynaptic folds are shown, with their subcellular localizations indicated by bars. Voltage gated sodium channels are localized in the depths of postsynaptic folds.

fusion of synaptic vesicles with the presynaptic membrane and the release of ACh. The post-synaptic membrane responds rapidly and dependably to ACh released from the overlying active zones in the nerve terminal. AChR, by binding ACh, become transiently permeable to both Na^+ and K^+, then opening the associated voltage-gated ion channels, which contribute to the action potential and muscle contraction. The synaptic cleft between nerve terminals and postsynaptic membrane is approximately 50 nm wide. A layer of connective tissue called basal lamina (basement membrane) sheaths each muscle fiber, passes through the synaptic clef and extends into the junctional folds. Both the presynaptic terminal and the muscle fiber secrete molecules including collagen IV, laminin, ectactin and heparan sulfate proteoglycans to the basal lamina. However, synaptic portions of the basal lamina contain their distinctive isoform composition separate from that of the extrasynaptic portions. Synaptic basal lamina also contain the enzyme acetylcholinesterase, which quickly inactivates the ACh released from the presynaptic terminal by hydrolyzing it to acetate and choline. Concentrations of released ACh in the synaptic cleft decrease rapidly by diffusion and interaction with acetylcholinesterase, upon which the neuromuscular transmission terminates.

Myasthenia gravis and autoantibodies to AChR

Myasthenia gravis (MG) is a rare neuromuscular disease, but a well-recognized disorder because of such characteristic clinical features as ptosis with fluctuating general fatigue and muscle weakness that worsens with

repeated activity,[1,2] but tends to improve with rest. Ptosis and diplogia occur early in the majority of these patients. With passing time, when the bulbar and respiratory muscles deteriorate, the disease becomes life-threatening so that intubation with mechanical ventilation is required. Approximately 80% of patients with MG have autoantibodies against AChR.[1,2] In 1973, Patrick and Lindstrom provided the first evidence indicating the pathogenicity of AChR antibodies in a model of experimentally induced MG.[3] Thereafter, a number of studies showed the pathogenic roles of AChR antibodies in causing structural and functional damage of the NMJ, but no such autoantigens could be identified in ~20% of these MG patients.[4] However, even patients who did not have AChR antibodies responded to immunotherapies, and their serum antibodies transferred a defect in neuromuscular transmission to mice, indicating that autoantibodies against NMJ can induce the muscle weakness.

Previously, studies on the mechanism(s) of synaptic transmission at the NMJ had facilitated understanding of how antibodies to AChR induce the pathogenicity typical of MG.[1,2] Effective neuromuscular transmission depends on numerous interactions between ACh and its receptor, AChR, and the failure of neuromuscular transmission results in myasthenic weakness and fatigue. To evoke action potential for the contraction of muscle fibers, a large enough number of AChR must be present at postsynaptic membranes. In 1973, Fambrough *et al.* found an abnormal decrease in the number of AChR at postsynaptic membranes of the NMJ of patients with MG.[5] Others showed that AChR antibodies affect neuromuscular transmission by three main mechanisms: (i) complement-mediated lysis of post-synaptic membrane by binding and activation of complement at the NMJ; (ii) accelerated degradation of AChR molecules cross-linked by antibodies (antigenic modulation); and (iii) functional AChR block by antibodies. The predominant pathogenicity is caused by the complement-mediated mechanisms,[6] but all three mechanisms tend to reduce the number of available AChR and, thereby, decrease neuromuscular transmission between motor nerve endings and postsynaptic membranes. Therefore, an individual nerve impulse cannot generate enough postsynaptic depolarization to achieve the crucial firing threshold required for opening of sufficient voltage-gated sodium channels to initiate an action potential in the muscle fiber.[7]

Antibodies to muscle-specific kinase in myasthenia gravis (MG) patients

For the last three decades, causative autoantibodies other than those to AChR have been sought in MG patients but have eluded identification despite extensive research efforts.[1,2] In 2001, Hoch *et al.* found autoantibodies against muscle-specific kinase (MuSK) in a proportion of patients with generalized MG.[4] MuSK is essential during the development of NMJ, when it organizes fetal AChR clustering at the postsynaptic membrane. Subsequently, in mature NMJ, MuSK is expressed predominantly at the postsynaptic membrane. Studies by Vincent *et al.* showed that the frequency of MuSK antibodies in "seronegative MG patients," that is those who lack autoantibodies to AChR, varied from 4 to 50%.[4,8–11] Ohta *et al.* detected MuSK antibodies in approximately 30% of seronegative MG patients but not in any MG patients with AChR antibodies (seropositive MG) or other autoimmune diseases.[12–14] The clinical features of patients with MG and MuSK antibodies are distinctive. These individuals often suffer from a severe bulbar dysfunction that is difficult to resolve with immunosuppressive and immunomodulatory treatments, and muscular atrophy of facial and tongue muscles is common.[14,15] The response to acetylcholine esterase inhibitors is generally unsatisfactory with the risk of worsening symptoms, especially when starting treatment in patients with bulbar symptoms or an impending respiratory crisis.[16] Thymectomy does not alleviate the symptoms.[14] In short-term therapy, patients with MuSK-positive MG respond as well to plasma exchange and intravenous immunoglobulin as those with AChR seropositive MG.[14] Even so, those patients whose neck and shoulder muscles are affected often experience respiratory weakness.[15] MG in which weakness is limited to the ocular muscle is not frequent but does occur.[15]

A number of clinical studies showed that MuSK MG constitutes a distinct subclass of the disease.[8–10,15] The reason is that many patients with MuSK antibodies develop severe muscle weakness and eventual atrophy, which is less common in patients with AChR seropositive MG, and the former respond differently to therapy than persons in the latter group. After the identification of MuSK antibodies in an MG patient, laboratory testing is now required to confirm the diagnosis of MG, to seek AChR antibodies and to formulate the clinical treatment.

MuSK functions in neuromuscular junctions (NMJ)

MuSK plays multiple roles in clustering AChR during development of the postsynaptic membranes of NMJ.[17,18] Contact of the motor-nerve growth cone with the muscle induces a narrow, distinct endplate zone in the mid-muscle that is marked by a high density of AChR clustering. In this step, agrin released from motoneurons activates MuSK and redistributes AChR clusters to synaptic sites.[18,19] However, agrin does not bind MuSK, and additional components are required to activate MuSK.[17,19] Recent studies showed that Lrp4,

a member of the LDLR family, is a receptor of agrin, forms a complex with MuSK and mediates MuSK activation by agrin.[20,21] Intriguingly, MuSK is also required for organizing a primary synaptic scaffold to create the post-synaptic membrane.[18] Prior to muscle innervation, AChR clusters form at the central regions of muscle fibers, creating an endplate zone that is somewhat broader than that in innervated muscle. Thus, MuSK is required for pre-patterning of AChR clustering in the absence of motor innervation. However, establishing a scenario for MuSK's participation in the process is somewhat complicated. For example, an element other than agrin might activate MuSK and trigger the postsynaptic specialization at NMJ. Simultaneously or alternatively, MuSK could act as a primary scaffold molecule without activation. The listed pleiotropic roles of MuSK in AChR clustering at developmental NMJ could also be required for the maintenance of mature NMJ.[22,23] Studies carried out *in vivo* have shown that synaptic AChR intermingle among themselves completely over a period of ~4 days and that many extra-synaptic AChR are incorporated into the synapse at the mature NMJ, although the synaptic membrane in adult muscle appears macroscopically to be stable.[24] Therefore, the mechanisms at play during AChR clustering in developing NMJ are also required in mature NMJ where postsynaptic complexes including those with AChR and MuSK are dynamically turning over for the maintenance of muscle function.

Do MuSK antibodies cause MG?

In contrast to the well-accepted mechanisms by which AChR antibodies function in MG, the pathogenic role of MuSK antibodies has been unclear.[25] First, no significant loss of AChR at NMJ was observed in biopsies from biceps brachii muscles of MuSK-positive patients with MG.[26] Second, MuSK antibodies are mainly in the IgG4 subclass, which does not activate complement[14] and complement-mediated damage to postsynaptic membranes, is considered a major source of pathogenicity in MG patients with AChR antibodies. Third, no research results have shown that passive transfer of MuSK serum from MG patients generates the equivalent disease in mice. Fourth, no experimental animal model of myasthenia gravis (EAMG) induced by immunization of MuSK protein has been developed. However MuSK antibodies from MG patients can inhibit MuSK functions *in vitro*.[4]

The pathogenicity of AChR antibodies was simulated experimentally by the induction of muscle weakness and development of paralysis in rabbits immunized with AChR protein purified from the electric eel.[3] This AChR protein induced the production of antibodies that cross-reacted with rabbit AChR at the NMJ. The flaccid paralysis that followed and electrophysiological studies

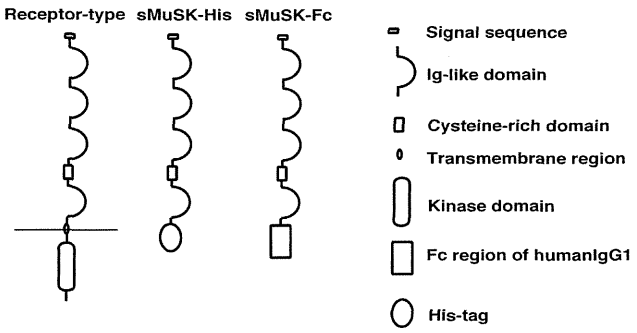


Figure 2 Schematic representation of the muscle-specific kinase (MuSK) domain structure and expression of secretory MuSK proteins in COS-7 cells. The domain structures of recombinant secretory MuSK protein (MuSK-His and MuSK-Fc) and receptor-type MuSK are shown. The whole coding region of the MuSK extracellular domain was fused with the His-tag or Fc region of human IgG1 as shown.

of these animals provided a model that resembled the MG of humans. Therefore, the demonstration of experimental autoimmune MG in animals induced by MuSK antibodies was essential for proving their pathogenicity and investigating their mechanisms of eliciting MG.

In 2006, we found that immunization of rabbits with MuSK ectodomain caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG,[23] as shown earlier by Patrick and Lindstrom.[3] The extracellular segment of MuSK comprised five distinct domains, that is four immunoglobulin-like domains and one cysteine-rich region (Fig. 2). The fusion protein expression constructs, which consisted of mouse MuSK ectodomain with the Fc region of human IgG1 or His-tag, were generated and transfected into COS-7 cells. The secreted recombinant MuSK-Fc and MuSK-His proteins were purified by using protein-A Sepharose and histidine affinity columns, respectively. New Zealand White rabbits were then immunized with 100–400 μg of purified MuSK recombinant protein. After three to four injections of MuSK protein, all six rabbits manifested flaccid paralysis (Fig. 3a). Sera from the paretic rabbits contained a high titer of MuSK antibodies that reacted specifically with MuSK molecules on the surfaces of C2C12 myotubes as observed in sera from MG patients who were positive for MuSK antibodies.[23] Histological studies of the muscle tissues from the paretic rabbits, which had manifested severe exhaustion, showed alterations in muscle fibers ranging from subtle to angular atrophy intermingled with normal muscle tissue (Fig. 3b). The histological changes typical of atrophied muscle fibers can result from MG, reduced mechanical ability or cachexia. In repetitive electromyograms from one of these paretic rabbits, the retroauricular branch of facial nerve was stimulated at 20 Hz, and recordings were taken from adjacent retroauricular muscle (Fig. 3c). The

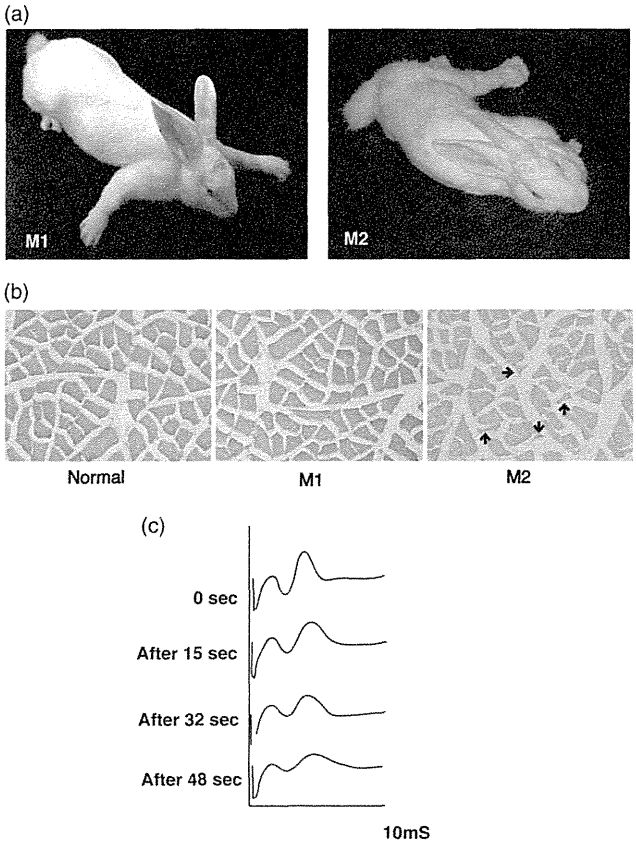


Figure 3 Rabbits manifest myasthenia gravis (MG)-like paresis after immunization with muscle-specific kinase (MuSK) protein. (a) Two rabbits representative of four animals with positive outcomes developed myasthenic weakness after immunization with the recombinant MuSK protein. After three injections of MuSK protein, M1 and M2 rabbits manifested flaccid weakness within 3 and 9 weeks, respectively. M2 rabbit developed severe exhaustion with muscle weakness. (b) Cross-sections from the soleus muscles of two paretic rabbits (M1 and M2) and a normal rabbit (Normal) were stained with hematoxylin–eosin. Muscle fibers in M1 paretic rabbit showed only subtle changes in shape and size, whereas atrophy of muscles fibers in M2 paretic rabbit was observed as small angular fibers (indicated by arrows; bar, 50 μm.) (c) Electromyograms recorded from M1 paretic rabbit. The retro-auricular branch of the facial nerve was continuously stimulated with constant square-wave pulses of 0.1 msec at 20 Hz delivered by a current stimulator, and the compound muscle action potential (CMAP; second peak observed on the oscilloscope screen recorded at the indicated time-points during stimulation) showed a decremental pattern, consistent with MG. Reproduced from *J Clin Invest* 2006; 116: 1016–1024 with permission. © 2009 The American Society for Clinical Investigation.

compound muscle action potential (CMAP) showed a decremental pattern, consistent with MG. However, injections of acetylcholine esterase inhibitor did not significantly reverse either the CMAP defect or the paralytic symptoms. Importantly, induction of myasthenia by

Figure 4 Manifestations of myasthenia gravis after injection of purified muscle-specific kinase proteins in a mouse.

MuSK antibodies is not confined to the rabbit, because we and others also produced myasthenia in mice by injection of MuSK protein (Fig. 4).[27,28]

How do antibodies to MuSK cause MG?

We have provided the first piece of evidence that active immunization with MuSK protein reproduces the MG-like disease in animals.[23,28] Next, we focused on how MuSK antibodies cause MG. The pathogenic role of MuSK antibodies in MG has been questioned, because the number of AChR is not reduced and complement is not deposited at the NMJ of biceps brachii muscles from MuSK-positive patients with MG.[26] The mechanisms used by AChR antibodies to cause MG are well delineated,[1,2] but those mechanisms simply do not apply to MG associated with MuSK antibodies. MuSK antibodies have been identified as predominantly IgG4, which does not activate complement. However, antibodies binding to MuSK could accelerate the degradation of MuSK molecules (antigenic modulation) and/or inhibit MuSK functions directly. MuSK is essential for AChR clustering at the developing NMJ, and its deficiency might lead to the complete loss of junctional ultrastructure.[22,29] Further, MuSK might also play important roles in the maintenance of AChR clustering and the structure of mature NMJ. To show precisely how MuSK antibodies participate in MG, unraveling the way in which MuSK acts at mature NMJ is necessary.

To elucidate the mechanisms of AChR clustering at NMJ, numerous studies were carried out using cultured C2C12 myotubes (Fig. 5). Agrin induces clustering of AChR in C2C12 myotubes after autophosphorylation by MuSK. *In vivo*, this event represents a major cascade of

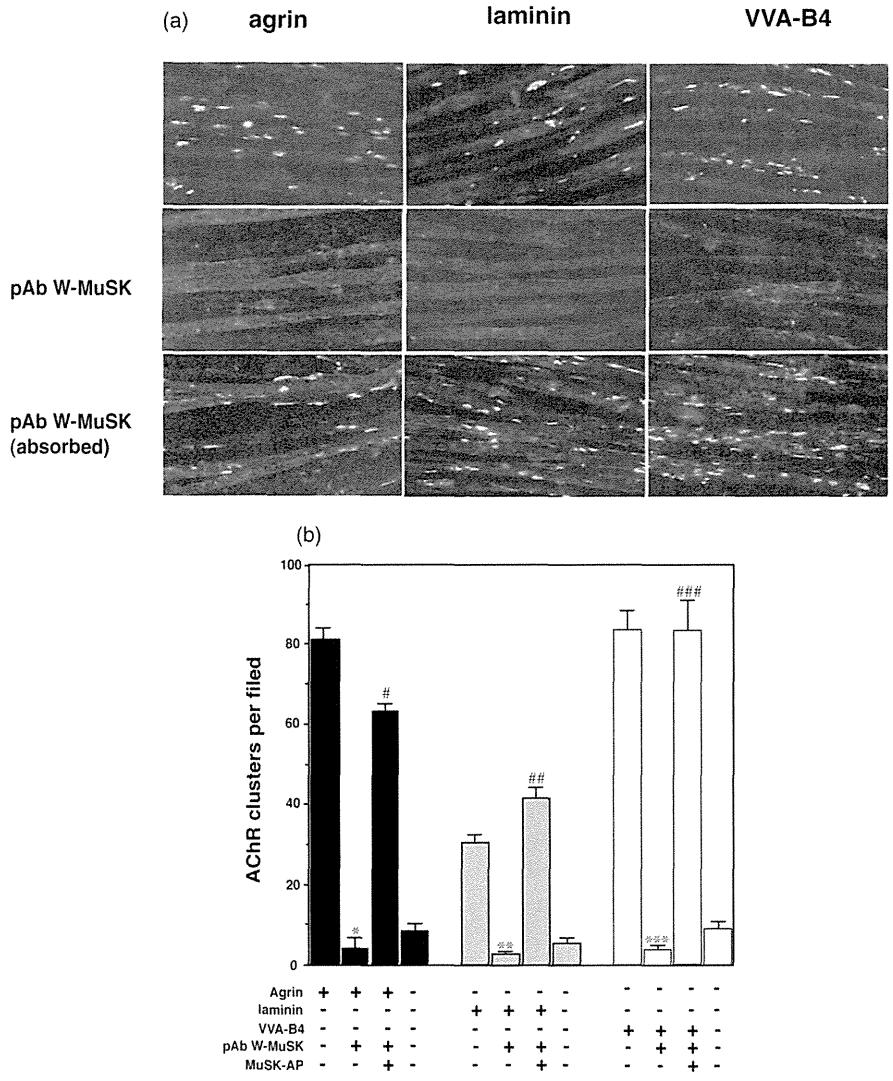


Figure 5 Inhibition of agrin-induced and agrin-independent acetylcholine receptors (AChR) clustering by muscle-specific kinase (MuSK) antibodies. (a) C2C12 cells were treated with agrin, laminin-1 or VVA-B4. AChR clusters were stained with rhodamine-conjugated BTX. AChR clustering induced by agrin, laminin-1 and VVA-B4 was inhibited in the presence of MuSK antibodies. This inhibition was blocked by absorption of the MuSK antibodies with MuSK-AP before treatment of the cells (bar, 20 μm). (b) Quantification of the inhibitory activity of the MuSK antibodies confirmed that they significantly inhibited agrin-, laminin-1– and VVA-B4-induced AChR clustering. Preabsorption of the MuSK antibodies with MuSK-AP significantly blocked inhibition. Values represent means ± SEM of 10–15 fields for each of the two experiments per treatment. $^{*,**,***}P < 0.01$ versus similar treatment without MuSK antibodies; $^{\#,\#\#,\#\#\#}P < 0.01$ versus similar treatment without preabsorption; ANOVA. *J. Clin. Invest.* 2006; **116**: 1016–1024. Copyright 2009 The American Society for Clinical Investigation.

AChR clustering at the NMJ after innervation by motoneurons. Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) also induce AChR clustering on C2C12 myotubes, without activation of MuSK. Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty. However, these mechanisms might also be important for the formation and maintenance of NMJ, the latter through agrin-independent pathways as shown by genetic studies.[23]

In a previous study, Hoch *et al.* observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering in C2C12 myotubes.[4] We also found that agrin-induced clustering of AChR was strongly blocked in the presence of MuSK antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect as shown in Figure 5.[23] These results showed that MuSK antibodies effectively inhibited the formation of agrin-induced AChR clustering. Intriguingly, the monovalent Fab fragments of MuSK antibodies from rabbits with

experimental autoimmune MG also inhibited AChR clustering by agrin on C2C12 cells, indicating that complement-mediated mechanisms are not necessarily required for such inhibition (unpubl. data). We also noted that MuSK-specific antibodies strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself (Fig. 5).[23]

We then examined the reduced expression of AChR at NMJ in soleus muscles of paretic and normal rabbits by using fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, α-BTX (Fig. 6). The use of a digital camera and staining with rhodamine-conjugated α-BTX enabled us to record the size and optical densities of AChR clusters. The resulting images were measured by using NIH image analysis software.[23] The areas and intensity of AChR fluorescence in muscles of these paretic rabbits were significantly reduced compared with those in normal rabbits. In addition, the structure of NMJ in our paretic rabbits as well as the size and branching of the motor terminals were significantly reduced. Electron microscopic observations of NMJ in rabbits with EAMG induced by injection of MuSK protein showed a significant loss of complexity in the convoluted synaptic folds but no destruction. A particularly important observation was that the EAMG model cited here resembles the phenotype of humans with MG and MuSK antibodies (Fig. 7). In the intricate and convoluted synaptic folds, the high density of voltage gated sodium channels in the membranes' depths amplify the end-plate current, thus enhancing neuromuscular transmission and muscle contraction.[30] A reduction in the size and branching of the motor terminals contributes to the reduced ACh output, and reduced post-synaptic folding increases the threshold for generation of muscle fiber action potential. These structural abnormalities in NMJ, including both pre- and post-synaptic structures, thus impair neuromuscular transmission in rabbits with EAMG.[28]

Intriguingly, similar abnormalities of NMJ structure were also observed in rats with reduced expression of MuSK as noted by RNA interference,[22] in a patient with congenital myasthenic syndromes (CMS) caused by MuSK mutations and also in mice expressing the MuSK missense mutation seen by electroporation experiments.[31] MuSK knockout mice also displayed presynaptic defects in addition to postsynaptic defects, indicating that MuSK is required for retrograde signals, so far unidentified, to maintain the presynaptic structure in mature NMJ.

Dok-7 is required for the maintenance of NMJ

In 2006, a MuSK-interacting protein called Dok-7 was discovered[32] and identified as a member of the Dok family of cytoplasmic proteins. Dok-7 is postulated to have three main functional domains: (i) a pleckstrin homology (PH) domain, essential for membrane association; (ii) a phosphotyrosine-binding (PTB) domain involved in the Dok-7 induced activation of MuSK; and (iii) a large C-terminal domain containing multiple tyrosine residues. Dok-7 knockout mice showed a marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Thus, Dok-7 is essential for neuromuscular synaptogenesis through its interaction with MuSK.

Mutations in the Dok-7 protein cause the genetic form of limb-girdle myasthenia called CMS.[33] Some clinical features of these patients resemble those in the severe type of MG accompanied by MuSK antibodies.[34] Proximal muscles are usually more affected than those in distal regions, as evident in MuSK MG patients, and ptosis is often present. However, limb-muscle weakness is comparatively less severe. Previous studies showed no reduction of AChR clustering with significant changes in NMJ of MuSK MG patients,[26] but further structural analysis of NMJ is required in muscles where severe weakness occurs commonly. The weakness and atrophy are not observed uniformly in muscles of these patients, although both MuSK and Dok-7 are essential for the formation of NMJ during the embryonic stage.[32] Notably, one of the major distinctions between acquired MuSK MG and CMS with the Dok-7 mutation is the timing when weakness begins. The CMS patients typically have difficulty in walking after reaching that normal motor milestone during early childhood, whereas the onset of weakness of MG patients, in most instances, occurs in adulthood. Interestingly, AChR clustering and post-synaptic folds are reduced and have small motor terminals as observed at NMJ in patients with CMS and Dok-7 mutations.[35] The effect of Dok-7 mutations on post-synaptic structures might also be an alteration of retrograde signaling to the pre-synaptic nerve terminals resulting in a reduced NMJ size in these patients (Fig. 7). Dok-7, along with MuSK, is also required for the maintenance of NMJ, not only for synaptogenesis.

MuSK plays important roles in the maintenance of NMJ

We have shown that MuSK is required for the maintenance as well as the generation of NMJ.[23,28] Disruption of those mechanisms by MuSK antibodies causes MG in humans. Use of an experimental model for MG showed that MuSK antibodies mediate the pathogenesis of this syndrome in rabbits and mice.[23,27,28] In most cases, the symptoms take more than 3 months to manifest themselves in rabbits and more than 4 weeks in mice. Furthermore, the symptoms were also induced