Biochemical and Biophysical Research Communications 424 (2012) 538–543

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Src kinase-mediates androgen receptor-dependent non-genomic activation of signaling cascade leading to endothelial nitric oxide synthase

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ARTICLE INFO

Article history:
Received 14 June 2012
Available online 6 July 2012

Keywords:
Testosterone
Androgen receptor
Non-genomic action
c-Src
eNOS
phosphatidylinositol 3-kinase/Akt

ABSTRACT

Our previous study has demonstrated that testosterone rapidly activates endothelial nitric oxide synthase (eNOS), enhancing nitric oxide (NO) release from endothelial cells (ECs) via the phosphatidylinositol 3-kinase/Akt (PI3-kinase/Akt) pathway. The upstream regulators of this pathway are unknown. In this study, we further investigated the non-genomic action of testosterone in human aortic ECs. Acute (30 min) activation of eNOS caused by testosterone was unaffected by pretreatment with a transcriptional inhibitor, actinomycin D. Non-permeable testosterone-BSA rapidly induced Akt and eNOS phosphorylation. In contrast, luciferase reporter assay showed that the transcriptional activity of the androgen-responsive element (ARE) was increased by testosterone, but not by testosterone-BSA at 2 h after stimulation. Immunostaining displayed co-localization of androgen receptor (AR) with caveolin-1. Fractional analysis showed that AR was expressed in caveolae-enriched membrane fractions. Immunoprecipitation assays revealed the association of AR with caveolin-1 and c-Src, suggesting complex formation among them. Testosterone rapidly increased the phosphorylation of c-Src on Tyr416, which was inhibited by an AR antagonist and by siRNA for AR. PP2, a specific-inhibitor of Src kinase, abolished the testosterone-induced phosphorylation of Akt and eNOS. Our data indicate that testosterone induces rapid assembly of a membrane signaling complex among AR, caveolin-1 and c-Src, which then facilitates activation of the c-Src/ PI3-kinase/Akt cascade, resulting in activation of eNOS.

1. Introduction

Steroid hormones play various roles in vascular functions through specific receptors localized in vascular endothelial cells (ECs) or vascular smooth muscle cells [1,2]. The production of the vasodilator nitric oxide (NO) by endothelial NO synthase (eNOS) is a key mediator of endothelial homeostasis, including normal vasomotor function [3]. Several lines of evidence indicate that testosterone can exert acute endothelium-dependent vasodilator effects upon various vascular beds and tissue perfusion throughout the body [4,5], and that these effects of testosterone are mediated in part by NO [6]. However, the detailed mechanisms of rapid vasodilatation by testosterone are unknown.

The androgen receptor (AR) is a member of the steroid nuclear receptor super-family, which exerts its effects by modifying gene expression [7–9]. In addition to its canonical genomic action, AR also exhibits acute actions, designated as non-genomic actions, which take place in a membrane-delimited signal pathway, taking only several seconds to minutes [10–12]. In vascular ECs, however, the range of signal transduction pathways activated by membrane AR has not been defined, and the potential roles of these pathways to mediate testosterone actions in vascular cells are largely unknown.

In vascular ECs, it is now established that eNOS and other regulatory proteins are co-localized in specialized signal-transduction plasma membrane domains, caveolae [13,14]. The localization of eNOS in caveolae is required for its activity [15,16]. We previously have demonstrated that in vascular ECs, testosterone acutely stimulates rapid eNOS activation and enhances NO production via activation of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt

Abbreviations: AR, androgen receptor; ARE, androgen-responsive element; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; PBS, phosphate buffered saline; PI3-kinase, phosphatidylinositol 3-kinase; testosterone-BSA, bovine serum albumin-coupled testosterone; NO, nitric oxide; siRNA, small interference RNA; NT-siRNA, non-targeting scrambled siRNA; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*] pyrimidine.

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http://dx.doi.org/10.1016/j.bbrc.2012.06.151

cascade driven by a direct interaction between AR and the p85α subunit of PI3-kinase [17]. In the present study, we further investigated whether testosterone activates eNOS via a membrane-AR mechanism, i.e., a non-genomic action, in human aortic ECs. We also investigated the upstream regulators involved in eNOS activation in the caveolae molecular complex.

2. Materials and methods

2.1. Cell culture

Human aortic ECs were maintained in EBM-2 (Clonetics) medium supplemented with 10% FBS and a growth factor cocktail as previously described [17]. Cells were used for the present experiments in the 5th and 7th passages. In the experiments using bovine serum albumin-coupled testosterone (testosterone-BSA) (Sigma), testosterone-BSA was diluted in culture medium to the indicated concentrations, then mixed with dextran-coated charcoal (50 mg/ml) for 30 min at room temperature, centrifuged at 3000*g* for 15 min and passed through a 0.2-μm pore size filter to remove any potential contamination with free testosterone. In inhibitor experiments, inhibitors were added 60 min before cells were treated with testosterone (100 nM).

2.2. Transfection of plasmids and luciferase reporter assay

The pGL3 vector containing two copies of androgen-responsive element (ARE) upstream of the minimal thymidine kinase promoter ligated to a luciferase reporter gene (2× ARE-TK-pGL3-luc) was used for luciferase reporter assay as previously reported [18]. As an internal control of transfection efficiency, a renilla luciferase plasmid pRL-TK (Promega) was co-transfected. Cells were seeded in six-well plates in culture medium and grown until 50–60% confluence, then transfected with the 2× ARE-TK-pGL3-luc reporter plasmid and pRL-TK control prasmid using SuperFect transfection reagent (Qiagen) for 24 h according to the manufacturer's instructions. Then, cells were rinsed with Hank's balanced salt solution buffer (Sigma) once, starved and exposed to testosterone (100 nM) or testosterone-BSA (100 nM) for an additional 2 h. The amount of plamid DNA of 2× ARE-TK-pGL3-luc per well was adjusted up to 0.9 μg and that of pRL-tk was 0.3 μg, as an optimal microgram ratio of 2× ARE-TK-pGL3-luc to pRL-TK was determined to be 3:1. The optimal ratio of the total amount of plasmid DNA (μg) to SuperFect transfection reagent (μl) was determined to be 10:1 by supplemental experiments. Firefly and renilla luciferase activities were measured using a dual luciferase assay system (Promega) according to the manufacturer's instructions. The firefly luciferase values of each sample were normalized by renilla luciferase activity, and data were reported as relative light units.

2.3. Immunostaining

Cells were plated onto type I collagen-coated cover slides and grown in culture medium until confluence. Cells were then fixed in 2.5% paraformaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature. After washing with cold PBS 3 times, cells were blocked with 3% milk for 30 min to prevent nonspecific binding. Slides were then incubated with a primary antibody mixture of rabbit anti-AR (N-20) (1:50 dilution; Santa Cruz) and mouse anti-caveolin-1 (1:100 dilution; BD Transduction) overnight, and then washed three times with PBS followed by incubation with a secondary antibody mixture of Alexa-fluor 488 goat anti-rabbit secondary Ab (1:200 dilution; Invitrogen) and Alexa-fluor 555 anti-mouse secondary antibody (1:200 dilution; Invitrogen) for 2 h. Cells were then incubated for 5 min with DAPI (Dojindo) to stain nuclei. Slides were mounted using mounting medium (Dako), and visualized using an Olympus FV300 laser scanning confocal microscope.

2.4. Cell fractionation

Low-density, caveolae-enriched membrane fractions were isolated using a Caveolae/Rafts Isolation kit (Sigma) according to the manufacturer's instructions. Cells were grown in 60-mm dishes until confluence, harvested in ice-cold lysis buffer containing 1% Triton X-100 and protease inhibitor cocktail (1:100 dilution), and sonicated for 5 s. The resulting cell lysates were cleared by centrifugation for 5 min. Supernatants were mixed with OptiPrep gradient layers, and the mixtures were obtained by centrifugation at 200,000*g* for 4 h using a Beckman SW70.1Ti rotor, and then analyzed by collecting and numbering 9 fractions (each 1 ml) from the top of the tubes. These fractions were subjected to immunoblotting. The amounts of all fractions of the loaded samples were equalized before gel electrophoresis. OptiPrep density gradient and immunoblotting experiments were performed four times, and representative blots are shown.

2.5. Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting experiments were performed as previously described according to standard protocols [17]. Antibodies against AR (N-20) for immunoblotting, AR (441) (Santa Cruz) for immunoprecipitation, phospho-eNOS^{Ser1177} and eNOS/NOS type III (BD Transduction), phospho-Src^{Tyr416} (Cell Signaling), c-Src (B-12) (BD Transduction), caveolin-1 (Sigma) for immunoblotting and caveolin-1 (BD Transduction) for immunoprecipitation were used. In all immunoprecipitation and immunoblotting experiments, blots were performed three times, and representative blots are shown. In some experiments, densitometry analysis was performed using an image scanner and analyzing software (NIH image Ver. 1.61).

2.6. Small interference RNA (siRNA) transfection

siRNA duplex against AR (Santa Cruz; Accession No: sc-29204) was used for directed knock down of AR expression. Non-targeting scrambled siRNA (NT-siRNA) (Santa Cruz; Accession No: sc-37007) was used as control siRNA. Transfection of siRNA was performed as previously described [17].

2.7. Statistical analysis

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

3. Results

3.1. Testosterone induced eNOS activation via non-transcriptional mechanisms

In our previous study, we demonstrated that ECs contain the AR, which is involved in phosphorylation of eNOS induced by testosterone [17]. To examine whether the response to testosterone is triggered through the nuclear receptor or initiated at the plasma membrane receptor in ECs, firstly, we investigated whether testosterone-induced activation of eNOS would be affected by exposure to actinomycin D (10 μM), a transcriptional inhibitor. As shown in Fig. 1A, acute (30 min) activation of eNOS caused by testosterone

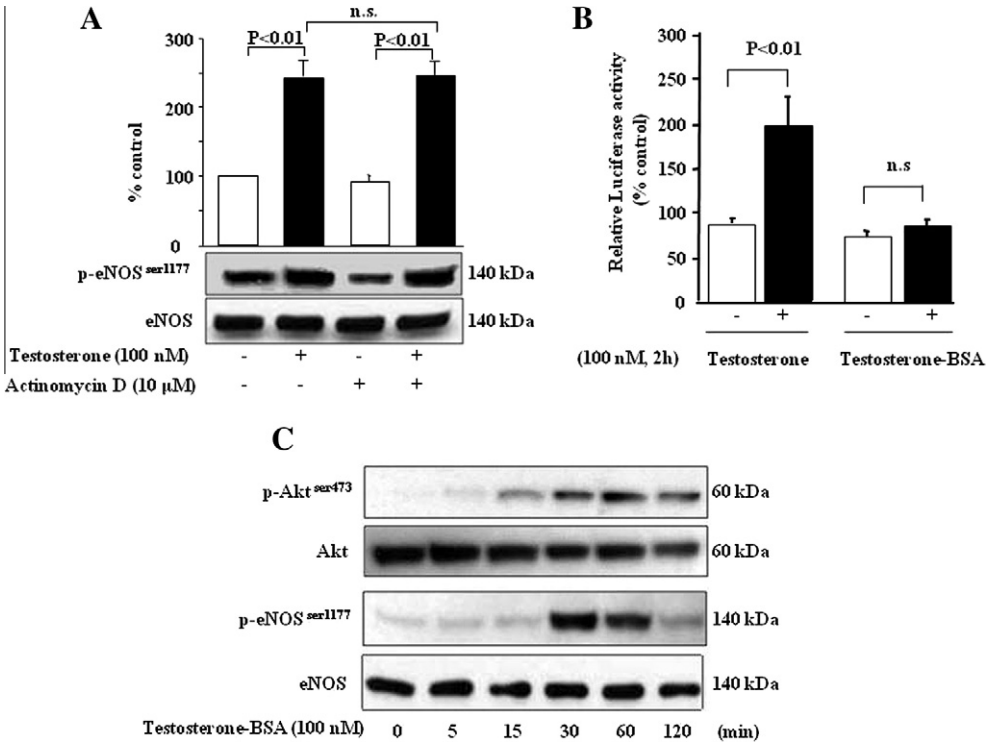

Fig. 1. Testosterone induced eNOS activation via non-transcriptional mechanisms. (A) Steroid-deprived, serum-starved ECs were pretreated with or without actinomycin D (10 μM) for 60 min before exposure to testosterone. Then, cells were treated with vehicle (0.01% DMSO) or testosterone (100 nM) for 30 min. Phosphorylation of eNOS at Ser1177 (p-eNOS^{Ser1177}) and the total eNOS level in cell lysates were analyzed by immunoblotting and densitometric analysis. Data represent mean ± SEM of the p-eNOS/eNOS ratio of quantified densities from three independent experiments. Representative blots are shown. (B) Luciferase reporter assay of ARE. Cells were transfected with the indicated plasmids in culture medium for 24 h, then starved and exposed to testosterone or testosterone-BSA for 2 h. After cells had been washed and harvested, cell lysates were prepared and used for luciferase reporter assay as described in Section 2. The results were obtained from three sets of transfection and are presented as mean ± SEM. $n = 6$. (C) Cells were treated with testosterone-BSA or vehicle for the indicated times. Phosphorylation of Akt and eNOS or the total levels of Akt and eNOS in cell lysates were analyzed by immunoblotting. The experiments were performed at least three times with comparable results.

was unaffected by pretreatment with actinomycin D. Then, we used membrane-impermeable testosterone-BSA to investigate whether testosterone triggered eNOS activation is mediated by the cell membrane AR. In the luciferase reporter assay, transcriptional activation of a specific DNA-binding response element, ARE, was increased when the cells were exposed for 2 h to testosterone, but not by exposure to testosterone-BSA (Fig. 1B). In contrast, testosterone-BSA rapidly induced Akt and eNOS phosphorylation (Fig. 1C), as is the case of testosterone [17]. These data indicate that testosterone rapidly activates eNOS via non-transcriptional mechanisms.

3.2. AR was distributed to caveolae in response to testosterone

As a signal complex scaffold protein, caveolae have been postulated to organize and modulate signal output [14,19]. Although AR is predominantly expressed in nuclei, confocal double immunostaining showed the co-localization of caveolin-1, a caveolae marker, and AR (Fig. 2A). In parallel, we performed cell fractionation with equilibrium density gradient centrifugation to determine whether AR is expressed in the caveolae-enriched membrane fractions. Caveolin-1 was highly concentrated in lighter fractions (Fig. 2B, #3 to #6 fractions), indicating that fractions 3–6 are the major caveolae-like plasma membrane microdomains under our experimental conditions. Although the majority of AR was deposited in the higher-density fractions (#7 to #9 fractions), a significant amount of AR was detectable in caveolin-1-enriched membrane fractions.

Next, we examined whether AR interacted with caveolin-1 directly in response to testosterone. As shown in Fig. 2C, the association of AR with caveolin-1 was detected in immunoprecipitation complex by immunoblotting reciprocally and significantly increased by exposure of cells to testosterone for 30 min, while the complex was detected at a low level without testosterone treatment. These results indicate that cells membrane AR was redistributed to the caveolin-1-enriched membrane fractions in response to testosterone.

3.3. c-Src kinase is a critical upstream regulator in PI3-kinase/Akt activation cascade

The Src family consists of nonreceptor tyrosine kinases that include nine members such as Src, Yes, Fyn, and c-Fgr. In some cells, Src is a critical upstream regulator of steroid-stimulated membrane signal transduction pathways [13,14]. In cell fractionation experiments, c-Src was present in the caveolin-1-enriched membrane fractions isolated from ECs (data not shown). We next investigated whether Src kinase mediates testosterone-induced eNOS activation in ECs. Phosphorylation of c-Src on Tyr416, the active form of c-Src, was increased after 5-min treatment of cells with testosterone (Fig. 3A). Pretreatment of cells with 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2; Sigma), a Src family kinase specific inhibitor, blocked the testosterone-induced phosphorylation of Src (Fig. 3A). PP2 also abrogated testosterone-stimulated Akt and eNOS phosphorylation (Fig. 3B). These data indicate that c-Src kinase is a critical upstream regulator of the Akt phosphorylation cascade for eNOS activation in ECs.

Testosterone-induced phosphorylation of c-Src was abolished when AR was knocked down by transfection with AR siRNA (Fig. 4A). Similarly, pretreatment with nilutamide, an AR antago-

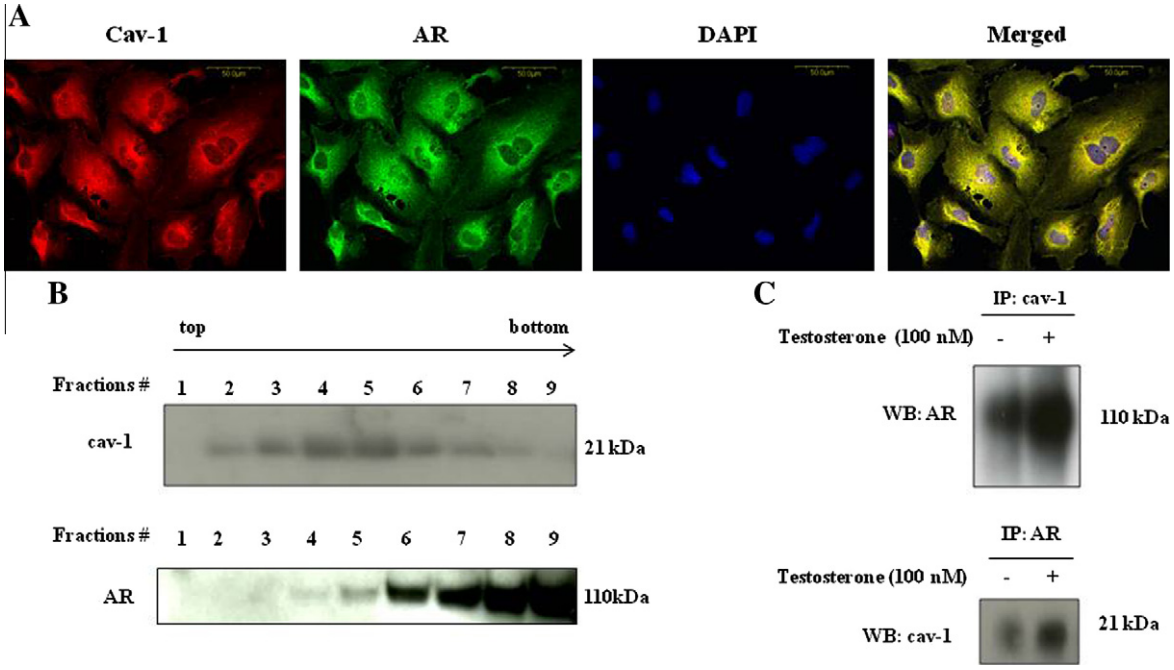

Fig. 2. AR was co-localized with caveolin-1 (cav-1) and distributed to caveolae in response to testosterone. (A) Confocal immunofluorescent images of AR and caveolin-1 in EC. Images of cells fixed on slides were merged by confocal microscopy as described in Section 2. An antibody mixture of rabbit anti-AR (N-20) and mouse anti-caveolin-1 were used to detect the proteins in ECs, followed by appropriate secondary antibodies (*red*, for caveolin-1 and *green*, for AR). Cells were incubated for 5 min with DAPI to stain nuclei. "Merged" shows an overlay of the caveolin-1 (*red*), AR (*green*) and DAPI (blue) signals. (B) Distribution of AR in caveolin-1-enriched caveolae membrane domain fraction. Cell fractionation was carried out by OptiPrep density gradient centrifugation as described in Section 2, and then developed by immunoblotting. Representative data for immunoblotting of cell fractionation from the tubs top fraction 1 to the bottom fraction 9 are shown. (C) Direct interaction of AR with caveolin-1 (cav-1). Cell lysates of ECs treated for 30 min with testosterone (100 nm) or vehicle were subjected to immunoprecipitation with antibodies against caveolin-1 or AR, separated by SDS–PAGE, detected with anti-AR or anti-caveolin-1 antibody, respectively. A representative result from three independent experiments is shown.

nist also abolished the testosterone-induced rapid phosphorylation of Src (Fig. 4B). Furthermore, immunoprecipitation assay revealed the association between AR and c-Src, which was increased by exposure of cells to testosterone for 30 min (Fig. 4C). Together with above-mentioned immunoprecipitation results about AR and caveolin-1, these data suggest that a complex formation among AR, c-Src and caveolin-1 in the plasma membrane was involved in testosterone-induced rapid eNOS activation.

4. Discussion

In the present study, we demonstrate that a caveolae-localized, AR-centered, multi-molecular complex is critical in rapid membrane-initiated eNOS activation induced by testosterone. Src kinase was shown to be an upstream regulator of the Akt and eNOS activation pathways.

Molecular, cellular and animal studies convincingly demonstrate that sex steroid hormones have various effects on vascular cells, and that many of these effects are achieved through rapid, membrane-initiated receptor-dependent signaling responses, which are different from the classical genomic actions [20]. AR is expressed in vascular ECs [17,21], and a number of reports have indicated that testosterone appears to have very rapid effects on the vascular system, including vasodilatation [22,23]. It has been shown that physiological concentrations of testosterone causes acutely (in minutes) NO-dependent vasodilatation via AR-mediated eNOS activation [24,25], which is consistent with the nongenomic nature of the response in arteries and intact ECs [26,27]. In the present study, we further demonstrated the signaling cascade driven from activation of membrane AR, which may explain mechanisms underlying rapid effects of testosterone on the vascular system.

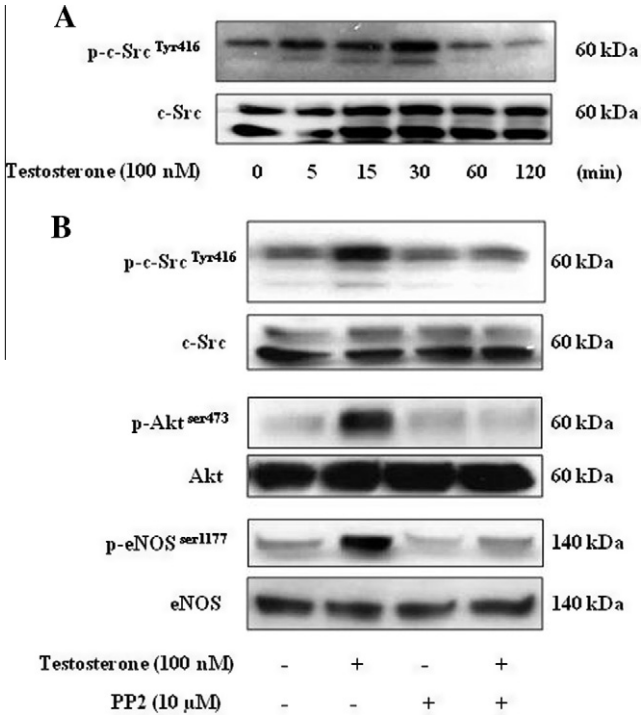

Fig. 3. Src kinase is a critical upstream regulator of Akt/eNOS activation pathway. (A) Steroid-deprived, serum-starved EC were treated with testosterone (100 nM) or vehicle for the indicated times. (B) PP2 (10 μM) was added 60 min before cells were treated with testosterone (100 nM) for 30 min. (A and B) Phosphorylation of c-Src kinase, Akt and eNOS (p-Src^{Tyr416}, p-Akt^{ser473}, p-eNOS^{ser1177}) and their total protein levels were analyzed using immunoblotting. A representative blot of three independent experiments with comparable results is show.

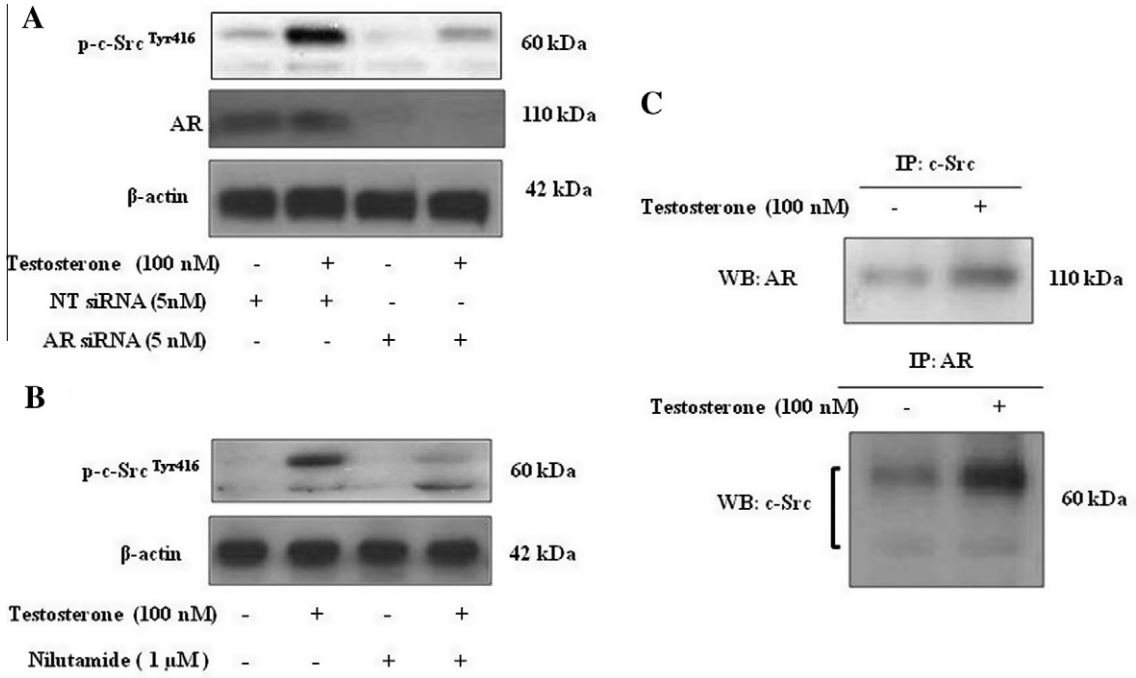

Fig. 4. The interaction between AR and c-Src. (A) Steroid-deprived, serum-starved cells were treated with testosterone or vehicle for 30 min after transfection of siRNA for AR or non-target siRNA (NT-siRNA). (B) Nilutamide (1 μM) was added 60 min before cells were treated with testosterone (100 nM) for 30 min. (A and B) Phosphorylation of c-Src kinase and AR or β-actin were analyzed using immunoblotting. A representative blot of three independent experiments with comparable results is show. (C) Cell lysates of EC treated for 30 min with testosterone (100 nM) or vehicle were subjected to immunoprecipitation with antibody against caveolin-1 or AR, separated by SDS–PAGE, detected with anti-AR or anti-caveolin-1 antibody, respectively. A representative result from three independent experiments is shown.

To further support the presence of functional membrane AR on EC, firstly we performed a series of experiments to compare the effects of the membrane-impermeable testosterone analog, testosterone-BSA with those of testosterone. Testosterone-BSA has been widely uses as a selective membrane AR ligand to study non-genomic actions of testosterone. Using testosterone-BSA and a transcriptional inhibitor actinomycin D, we showed that the rapid effect of testosterone on eNOS activation is independent of nuclear transcription activities, and that plasma membrane AR is responsible for this signaling pathway, similar to the non-genomic actions of other steroids such as estrogens [13,27,28]. In vascular ECs, caveolae are identifiable plasma membrane invaginations. Caveolin-1 is a caveolar structural protein with a long intramembrane domain, which directs caveolae targeting of multiple signaling molecules including Src family tyrosine kinases, PI3-kinase and steroid sex hormone receptors [28–31]. In the present study, confocal images and cell fractionation experiments confirmed the colocalization of AR and caveolin-1 in the plasma membrane, providing an evidence for above-mentioned non-genomic action of testosterone in EC.

The location-sensitive, membrane-associated non-receptor tyrosine kinase, Src, plays a physiological role in vascular function including vasorelaxation [13,27]. Others have indicated that Src activates PI3-kinase through steroid hormone receptors as a signaling cascade [32,33]. In the present study, c-Src interacted directly with AR in the caveolin-1-enriched membrane domain. Testosterone stimulated rapidly c-Src phosphorylation on Tyr416 which was abolished by an AR antagonist and by transfection of AR siRNA. Furthermore, a Src kinase specific inhibitor blocked the increase in phosphorylations of c-Src, Akt and eNOS. Taking these results together, we can conclude that a sequential cascade, AR-initiated c-Src/PI3-kinase/Akt activation is mediated in testosterone-induced rapid eNOS action in EC.

In summary, we demonstrated that testosterone induces the rapid assembly of a membrane-initiated signaling complex among AR, c-Src and caveolin-1, which facilitates activation of c-Src/PI3-kinase/Akt cascade with consequent activation of eNOS in vascular ECs. These findings support the concept of rapid membrane-initiated testosterone responses in the vascular endothelial system, and may provide evidence or an explanation for the favorable effects of testosterone on vascular function.

Disclosure statement

The authors have nothing to disclose.

Acknowledgment

We thank Prof. Shigeaki Kato (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan, 113-0032) for providing ARE plasmid.

Grants

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan (21390220, 20249041, 24390180).

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