Role of BK_{Ca} and SK_{Ca} channels in testosterone-induced nitric oxide release from human coronary artery endothelial cells

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Abstract

Testosterone is suggested to have beneficial and protective roles on cardiovascular system including vasodilation and lowering of blood pressure. Testosterone has been suggested to induce nitric oxide (NO) release from endothelial cells of different vascular origins. Information from previous studies provided that testosterone induces activating phosphorylation of endothelial nitric oxide synthase (eNOS) by acting through surface androgen receptor (sAR) and activating Src/PI3K/Akt-dependent pathway. A recent electrophysiological study in our laboratory using whole-cell patch-clamp technique demonstrated that testosterone-induced current mainly passed through large- and small-conductance Ca^{2+}-activated K^+ (BK_{Ca} and SK_{Ca}, respectively) channels. However, the involvement of BK_{Ca} and SK_{Ca} channels in testosterone-induced NO release was not elucidated. We herein examined the effect of testosterone-induced NO release and part of its mediators. We demonstrated that testosterone achieves its effect by acting through sAR and causes a dose-dependent increase in NO release from human coronary artery endothelial cells (HCAECs). This enhanced NO release was not affected by inhibitors of $G\alpha_{i/o}$ or PKA. Interestingly, although inhibitors of either BK_{Ca} or SK_{Ca} channels did not alter the testosterone-induced NO release, the combination of both BK_{Ca} and SK_{Ca} channel blockers significantly abolished the effect. These data suggest that testosterone induces NO release from HCAECs and its Ca^{2+}-dependent pathway requires either BK_{Ca} or SK_{Ca} channel to function properly.

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Introduction

Cardiovascular disease (CVD) is one of major health problems in both genders.[1] Existence of sexual dimorphism led to the idea that testosterone, a male sex hormone, exerts detrimental effects on cardiovascular system (CVS).[2-4] Recent evidence, however, suggested that testosterone produces beneficial cardioprotective effects.[5,6] Testosterone was shown to induce coronary vasodilation, enhance coronary perfusion, and increase angina threshold.[7-9] Testosterone has been shown to achieve its vasodilatory effect through both endothelial-dependent and -independent manners in various types of vessels.[10-12]

Both vascular endothelial cells and smooth muscle cells contain functional androgen receptors and play significant roles in maintaining physiological functions of vessels.[13] Human coronary artery endothelial cells (HCAECs) produce several vasoactive substances including nitric oxide (NO), endothelial-derived hyperpolarization factor (EDHF), prostacyclin (PGI_2), and endothelin.[14,15] Testosterone is suggested to induce NO release from endothelial cells of various vascular origins, although the specific mechanism is still unclear.[16-19]

Nitric oxide synthase (NOS) is an enzyme that when activated converts L-arginine to L-citrulline and NO by utilizing molecular oxygen in the presence of cofactors: tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN).[20-23] Among three isoforms of nitric oxide synthase, endothelial NOS (eNOS) is the major source of endothelial-derived NO.[24] Induction of NO production by eNOS from vascular endothelial cells can be generally divided into Ca^{2+}-dependent and -independent mechanisms.[25]

Although the detailed mechanisms of testosterone-induced NO release have not been clearly determined, multiple putative pathways have been proposed. Information from previous studies stated that testosterone-induced endothelial NO release is mediated through surface androgen receptor (sAR) located in membrane caveolae which subsequently activates PI3K/Akt pathway and induces eNOS activation[17,19] with proto-oncogene tyrosine-protein kinase Src (c-Src) as its upstream regulator.[18] A recent study in our laboratory using

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whole-cell patch clamp technique showed that testosterone, at both physiological and supraphysiological concentrations, induced currents through large- and small-conductance Ca^{2+}-activated K^+ (BK_{Ca} and SK_{Ca}, respectively) channels and this effect involved $G\alpha_{i/o}$/PKA pathway.[12] Our result, however, could not ascertain the involvement of other intracellular pathways and second messengers. Moreover, our previous study did not directly measure the actual amount of testosterone-induced NO release from HCAECs.

The aim of this study is to examine the acute effect of testosterone on NO release from HCAECs and part of its signaling pathway. We confirmed the testosterone effect on NO release using different forms of testosterone. We then examined some of the mediators involved using blockers and NO release assay. Our results illustrated the effect of testosterone on NO release from HCAECs and the significance of BK_{Ca} and SK_{Ca} channels in this process.

Materials and Methods

Cell culture

Commercially available HCAECs (Lonza, Walkersville, MD, USA) were used in all experiments. The cells were cultured in medium containing endothelial cell basal medium containing growth supplements (0.4% bovine brain extract, 0.1% human endothelial growth factor, 0.1% hydrocortisone, 30 μg/ml gentamycin sulfate, 15 ng/ml amphotericin-B, and 5% fetal bovine serum) (EGM™-MV Bullet Kit™, Lonza). The culture was kept at 37°C in humidified chamber with 5% CO_2. The medium was changed every 2-3 days. Subculturing was done when the cells reached 80% confluency. The cells from 5^{th}-7^{th} passages were used in all experiments. Twenty-four hours before each experiment, HCAECs were incubated with phenol red-free medium containing 2% fetal bovine serum.

Reagents

Testosterone, dihydrotestosterone (DHT), bovine serum albumin-conjugated testosterone (T-BSA), pertussis toxin (PTX), N-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide (H-89), apamin (Apa), Griess reagent, and nitrate reductase were obtained from Sigma-Aldrich (St. Louis, MO, USA). Iberiotoxin (IbTX), 4-amino-5-(4-chlorophenyl)-7-(dimethyl-ethyl)pyrazolo[3,4-d]pyrimidine (PP2), and colorimetric nitric oxide assay kit were obtained from Abcam (Cambridge, UK). Testosterone, T-BSA, and DHT were dissolved in ethanol (final concentration not exceeding 0.1% v/v). PTX, H-89, and IbTX were dissolved in water. PP2 was dissolved in dimethyl sulfoxide (DMSO). Apa was dissolved in 5% acetic acid. The agonists and blockers were reconstituted to their working concentration in phenol red-free medium containing 2% fetal bovine serum prior to each set of experiments.

NO measurement

After each respective treatment, the medium was collected and centrifuged at maximum speed for 5 minutes at 4°C. NO level in the supernatant was measured by using NO colorimetric assay. Briefly, nitrate in the sample was converted back to nitrite by adding assay buffer together with nitrate reductase. After at least 30 minutes of incubation, enhancer was added and incubated for 10 minutes. Griess reagent was then added to convert nitrite to an azo compound with deep purple color which can be detected by absorbance measurement at 540 nm. The total nitrite concentration in each sample was calculated by using a graph of nitrite standard with known concentrations. The results were reported as percentage of change compared to their respective controls.

Effect of testosterone on NO release

Various testosterone concentrations were used for the experiment to test the testosterone effect on HCAEC NO release and to find the optimal dose to be used for subsequent experiments. HCAECs were incubated with 10, 30, 100, 300, 1000, or 3000 nM testosterone for 15 minutes followed by NO measurement. The resulted optimal dose was used in subsequent experiments.

A non-permeant form of testosterone, T-BSA, was used to test the involvement of sAR. A non-aromatizable form of testosterone, DHT, was used to exclude the possible estrogenic action of testosterone aromatization. HCAECs were incubated with T-BSA or DHT using the same dosage as the optimal dose of testosterone for 15 minutes then the NO release was determined.

Identification of signaling pathway involved

HCAECs were pretreated with specific combination of blockers, each with dosage and duration as follows: PTX (1 μg/ml, overnight), PP2 (10 μM, 1 hour), H-89 (25 μM, 1 hour), Apa (5 μM, 5 minutes), IbTX (0.5 μM, 5 minutes). After the blocker treatment, T-BSA was added and the cells were incubated for additional 15 minutes followed by the NO assay.

Statistical analysis

Data are expressed as mean ± SEM. Each set of experiment was repeated for 3-8 times. GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. The log-dose-response curve was plotted by using the dose-response equation function in the software. Data normality was tested using Kolmogorov-Smirnov test. One sample t-test was used to compare a group of data to a specific value (e.g., 100%). Two independent groups were compared using unpaired t-test. Analysis of variance followed by Dunnett's multiple comparisons was used for comparisons among three or more groups. A P value of less than 0.05 was considered statistically significant.

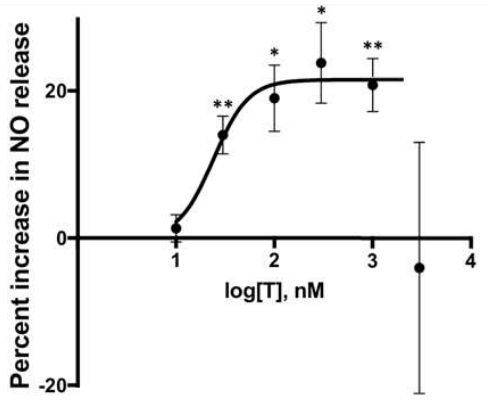


Figure 1 Testosterone dose-dependently increased NO release from HCAECs. The curve represents percent changes in NO release from HCAECs after 15-minute testosterone treatment (10, 30, 100, 300, 1000, and 3000 nM, respectively). The doses of 30-1000 nM induced significant increases in NO release compared to control (*P < 0.05, **P < 0.01, n = 5). The data from 3000 nM were excluded because of the inhibitory effect. Data are shown as mean ± SEM.

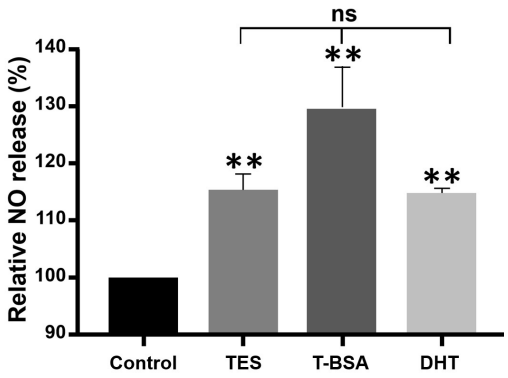


Figure 2 Testosterone acts through sAR to enhance NO release from HCAECs. Treatment of HCAECs with 100 nM of either testosterone, T-BSA, or DHT for 15 minutes could significantly enhance NO release compared to control (115.60 ± 2.47%, n = 7; 129.90 ± 7.04%, n = 7; 115.00 ± 0.58%, n = 3; respectively, **P < 0.01). Data are shown in mean ± SEM. ns = not significant.

Results

Testosterone rapidly and dose-dependently enhances NO release from HCAECs

To determine the effect of testosterone on NO release from HCAECs and to find its optimal dose, HCAECs from the 5th-7th passages were treated with testosterone (10, 30, 100, 300, 1000, or 3000 nM). After a 15-minute incubation, the amount of NO released from each condition was measured. Figure 1 represents the log-dose-response curve plotted from the data. Treatment of HCAECs with testosterone at concentrations of 30, 100, 300, and 1000 nM significantly increased NO release compared to control with mean percentage increases of 14.00 ± 2.57%, 19.00 ± 4.51%, 23.80 ± 5.47%, and 20.80 ± 3.63%, respectively (P < 0.05, n = 5). However, 10 nM testosterone caused no significant increase in NO release while the dosage of 3000 nM caused an inhibitory effect. Fitting the data to a dose-response curve equation yielded an EC50 of 24.1 ± 1.40 nM, a Hill coefficient of 2.46 ± 2.25, and 21.51 ± 2.43% maximum increase. The 100 nM concentration was chosen as the optimal dose to be used in the subsequent experiments.

Testosterone induces NO release in HCAECs through surface androgen receptor

To identify whether testosterone acts through sAR to enhance NO release from HCAECs, T-BSA, a non-permeant form of testosterone, at 100 nM was used to incubate the HCAECs for 15 minutes followed by NO release assay. As shown in Figure 2, T-BSA could also significantly enhance NO release from HCAECs (129.90 ± 7.04%) to a comparable extent with testosterone (115.60 ± 2.47%), P = 0.05. This suggests that testosterone effect on NO release is mediated through sAR.

Testosterone-induced NO release from HCAECs is mainly androgenic effect

To distinguish the androgenic effect of testosterone from its possible estrogenic effect due to aromatization into estrogen, dihydrotestosterone (DHT) which is a non-aromatizable form of testosterone was used. DHT at 100 nM could significantly enhance NO released form HCAECs compared to control (115.00 ± 0.58%) as shown in Figure 2. The effect was neither different from testosterone nor T-BSA, P = 0.22 and 0.18, respectively.

Testosterone can induce NO release via Ca^{2+}-dependent pathway

To study the Ca^{2+}-dependent activation of NO release by testosterone, a known Ca^{2+}-independent (c-Src/PI3K/Akt) pathway was blocked at their upstream regulator, c-Src, by PP2 before incubation with T-BSA. As shown in Figure 3, testosterone-induced NO release was significantly higher in PP2 treated group (178.60 ± 13.46%) compared to T-BSA

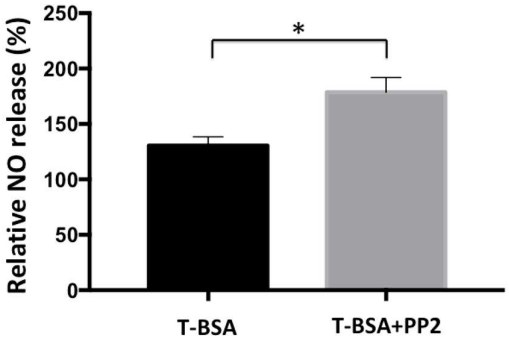


Figure 3 Testosterone induces NO release via a Ca^{2+}-dependent pathway. Pre-treatment with PP2 significantly enhanced NO release from HCAECs when T-BSA was given compared to T-BSA only group. (178.60 ± 13.46%, 130.60 ± 7.94% respectively), *P < 0.05, n = 5. Data are expressed as mean ± SEM.

only group (130.60 ± 7.94%), $P < 0.05$. These data indicate that testosterone can enhance NO release from HCAECs via a Ca^{2+}-dependent pathway. Moreover, we observed an enhancement of NO release when c-Src was blocked by PP2 which could be an indirect effect of c-Src inhibition.

$G\alpha_{i/o}$/PKA-dependent pathway is not essential in testosterone-induced NO release from intact HCAECs

The contribution of $G\alpha_{i/o}$/PKA-dependent pathway in testosterone-induced NO release from HCAECs was tested by using their specific blockers, PTX and H-89 for $G\alpha_{i/o}$ and PKA inhibition, respectively. Testosterone-induced NO release from HCAECs in the presence of PP2 (T-BSA+PP2: 178.60 ± 13.46%) was not significantly altered by blocking either $G\alpha_{i/o}$ (T-BSA+PP2+PTX: 203.00 ± 20.54%, $P = 0.87$), PKA (T-BSA+PP2+H89: 224.60 ± 21.47%, $P = 0.32$), or both $G\alpha_{i/o}$ and PKA simultaneously (T-BSA+PP2 +PTX+H89: 214.60 ± 18.00%, $P = 0.58$), as shown in Figure 4. These data suggest that $G\alpha_{i/o}$/PKA-dependent pathway is not crucial to the testosterone-induced Ca^{2+}-dependent NO release in intact HCAECs.

SK_{Ca} and BK_{Ca} channels are necessary for testosterone-induced, Ca^{2+}-dependent NO release

The roles of SK_{Ca} and BK_{Ca} channels in testosterone-induced NO release from HCAECs were tested by using their specific blockers, Apa and IbTX, respectively. As shown in Figure 4, pretreatment of HCAECs with either Apa or IbTX alone, in the presence of PP2, did not significantly alter testosterone-induced NO release (T-BSA+PP2: 178.60 ± 13.46% *vs.* T-BSA+PP2+Apa: 182.60 ± 19.47% *vs.* T-BSA+PP2+IbTX: 175.60 ± 16.84%, both $P > 0.05$ vs T-BSA+PP2). However, when both SK_{Ca} and BK_{Ca} channels were simultaneously blocked in the presence of PP2, the testosterone-induced NO release from HCAECs was significantly inhibited (T-BSA+PP2+Apa+IbTX: 105.00 ± 15.00%, $P < 0.05$ *vs.* T-BSA+PP2, T-BSA+PP2+Apa, and T-BSA+PP2+IbTX). These data suggest that when only one of either BK_{Ca} or SK_{Ca} channels was blocked, the other functional channel could compensate for the blocked channel. However, when both channels were blocked simultaneously, the compensation was lost and the testosterone-induced Ca^{2+}-dependent NO release pathway was suppressed suggesting the involvement of these two channels in Ca^{2+}-dependent NO release.

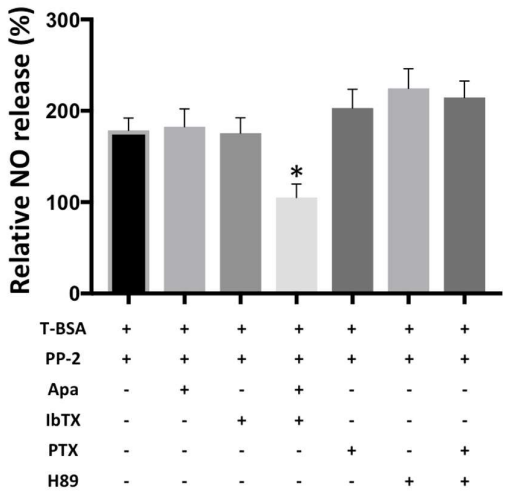


Figure 4 BK_{Ca} and SK_{Ca} are necessary for testosterone-induced NO release from HCAECs in Ca^{2+}-dependent manner. The NO release from HCAECs treated with T-BSA+PP2+Apa group, T-BSA+PP2+IbTX group, T-BSA +PP2+PTX group, T-BSA+PP2+H89 group, and T-BSA +PP2+PTX+H89 group (182.60 ± 19.47%, 175.60 ± 16.84%, 203.00 ± 20.54%, 224.60 ± 21.47%, 214.60 ± 18%, respectively), are not significantly different compared to T-BSA+PP2 group (178.60 ± 13.46%). However, NO release from T-BSA+PP2+Apa+IbTX group (105.00 ± 15%, n = 5) is significantly lower than that of T-BSA+PP2 group, $P = 0.04$. Data are expressed as mean ± SEM.

We further investigated the involvement of SK_{Ca} and BK_{Ca} channels in the testosterone-induced NO release from HCAECs in the absence of c-Src inhibitor, PP2. Both SK_{Ca} and BK_{Ca} channels were simultaneously blocked with their specific blockers, Apa and IbTX, respectively, followed by a 15-minute treatment with testosterone. Unlike the preceding result, as shown in Figure 5, simultaneous blockage of both SK_{Ca} and BK_{Ca} without concurrent inhibition of Ca^{2+}-independent NO release pathway did not significantly affect testosterone-induced NO release from HCAECs (T-BSA+Apa+IbTX, 129.83 ± 13.08% *vs.* T-BSA, 148.67 ± 12.93 %, $P = 0.33$, n = 6). These data suggest that the role of SK_{Ca} and BK_{Ca} channels in NO release can be compensated by an intact Ca^{2+}-independent pathway.

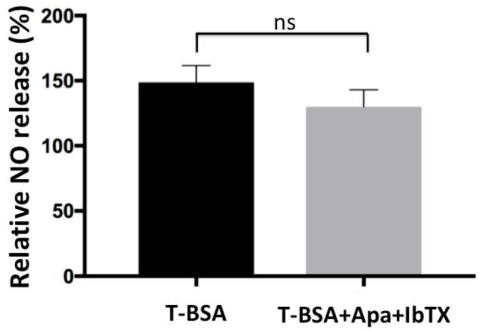


Figure 5 The role of BK_{Ca} and SK_{Ca} can be compensated by the intact Ca^{2+}-independent pathway. The NO release from HCAECs treated with T-BSA (148.67 ± 12.93%) is not significantly different from that of T-BSA+Apa+IbTX (129.83 ± 13.08%), $P = 0.33$, n = 6. Data are expressed as mean ± SEM.

Discussion

The present results demonstrated that a 15-minute exposure to physiological testosterone concentration could significantly enhance NO release from

HCAECs. Moreover, both T-BSA and DHT could significantly enhance NO release from HCAECs in a similar fashion as testosterone, suggesting the sAR involvement and the non-genomic action of testosterone. However, since blocking $G\alpha_{i/o}$/PKA-dependent pathway did not significantly affect testosterone-induced NO release from intact HCAECs, there might exist another pathway that is sufficient for the same effect. Although, there was no significant inhibitory effect when either BK_{Ca} or SK_{Ca} was blocked, simultaneous blockage of BK_{Ca} and SK_{Ca} in the presence of PP2 significantly disrupted the testosterone-induced NO release from HCAECs emphasizing the importance of BK_{Ca} and SK_{Ca} in regulating Ca^{2+}-dependent pathway. On the other hand, when both BK_{Ca} and SK_{Ca} were simultaneously blocked without inhibiting Ca^{2+}-independent pathway, there was no more significant blockage suggesting that Ca^{2+}-dependent pathway can be compensated by Ca^{2+}-independent pathways. Our results contribute new details regarding mechanisms underlying how testosterone induces NO release from endothelial cells and the involvement of certain signaling components.

Testosterone was previously believed to have detrimental effects on the CVS as opposed to beneficial effects conferred by estrogen.[26-31] Overtime, accumulating evidence suggests cardiovascular beneficial effects elicited by testosterone[5,32] including acute vascular relaxation[33-38] and restoration of normal vascular function.[39,40] Moreover, low levels of testosterone have been observed in male population with hypertension[41-43] and coronary heart disease[44-49] leading to the notion that having low levels of testosterone is a cardiovascular risk factor.[44,50,51]

Testosterone has been found to decrease blood pressure, dilate coronary vessels, improve coronary perfusion and increase angina threshold.[7-9] Although the specific mechanism of testosterone-induced acute vasorelaxation is still unclear, many studies suggested the involvement of NO.[9,33,36,52] NO is a well-known signaling molecule which regulates cardiovascular function.[53-55] Testosterone has been reported to induce NO release from endothelial cells of different vascular origins such as rat blood vessels,[16] human umbilical vein,[17] and human aorta.[18,19] Nonetheless, the effect of testosterone on NO release from HCAECs was still unknown.

HCAECs produce various vasodilatory substances including NO.[14,15] Several ion channels are present in HCAECs including Cl^- channels, Ca^{2+}-permeable non-selective cation channels, and various types of K^+ channels.[56] These channels are known to play important roles in regulation of vasoactive substance releases by changing the membrane potential and altering the electrical driving force for Ca^{2+} entry.[57,58]

Many studies suggested that K^+ channels play a major role in the regulation of endothelial membrane potential.[59] Several K^+ channels are found in HCAECs, namely large-, intermediate-, and small-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}, IK_{Ca}, and SK_{Ca}), inwardly rectifying K^+ (K_{ir}) channels and ATP-sensitive K^+ (K_{ATP}) channels.[56] Opening of K^+ channels causes net K^+ efflux which hyperpolarizes the cell membrane and enhances the driving force of Ca^{2+} influx. Increased intracellular Ca^{2+} concentration in endothelial cells increases NO release[60] by binding of Ca^{2+} to calmodulin (CaM) and eNOS activation by the Ca^{2+}-CaM complex.[61,62]

Among three forms of NOSs, eNOS is the major source of endothelium-derived NO[24] catalyzing the conversion of L-arginine to L-citrulline and producing NO when activated.[63-65] eNOS has been found to be activated by several mechanisms including Ca^{2+}-dependent dissociation from inhibitory interaction with caveolin-1 (Cav-1),[64,66-68] interaction with heat shock protein 90,[66-68] dimer formation to bind with its cofactor BH_4 and substrate L-arginine,[64,68-72] phosphorylation at the activation site (Ser1177),[73-77] and dephosphorylation at the negative regulatory site (Thr495).[74]

Multiple putative pathways for testosterone-induced NO release from endothelial cells have been proposed but their detailed mechanisms have not yet been clearly elucidated. These pathways can be generally divided into two main groups, namely Ca^{2+}-dependent and Ca^{2+}-independent activation.[25] Testosterone induces endothelial NO release independent of Ca^{2+} by acting through sAR, with c-Src as its upstream regulator, to activate eNOS either via PI3K/Akt pathway[18,19] or ERK1/2 phosphorylation of eNOS at Ser1177.[17] For Ca^{2+}-dependent pathway, binding of mediators to receptors causes a change in calcium dynamics inside the endothelial cells resulting in intracellular Ca^{2+} increase[64,72,78] and Ca^{2+}-CaM complexes which activates eNOS,[61,62] by freeing eNOS from caveolin-1 inhibitory clamp.[64,66-68] The free eNOS then can be activated and produce NO.[79]

A recent electrophysiological study from our lab using whole-cell patch-clamp technique to study HCAECs found that testosterone, at both physiological and supraphysiological concentrations, enhanced ionic currents through BK_{Ca} and SK_{Ca} channels, which could be attenuated by blocking $G\alpha_{i/o}$/PKA-dependent pathway.[12] These results suggest the involvement of BK_{Ca} channels, SK_{Ca} channels, and $G\alpha_{i/o}$/PKA-dependent pathway in enabling testosterone action, but could neither exclude the involvement of other pathways nor demonstrate the final effect on testosterone-induced NO release from HCAECs by this ion channel-mediated pathway.

Therefore, in this study, we examined the acute effect of testosterone on NO release from HCAECs and part of its signaling pathways by treating HCAECs with testosterone for 15 minutes in combination with specific blockers. Phenol red-free media containing only 2% fetal bovine serum was used to avoid possible estrogenic activity from phenol red. Our result of testosterone-induced NO release agrees with those from previous studies done in endothelial cells of various vascular origins, albeit with different

magnitude of NO releases.[17-19] Campelo and Goglia both demonstrated, in agreement with the current results, that low doses of testosterone significantly enhance NO release while higher doses cause a decrease in NO release from endothelial cells.[16,17]

Sex steroids can act on vascular cells in a rapid, membrane-initiated, receptor-dependent signaling pathways in addition to the canonical genomic pathway.[80] sAR is expressed in endothelial cells[19,81] and is a target of hormones.[13] In the current study, T-BSA, a membrane-impermeable form of testosterone, could enhance HCAEC NO release to a level similar to an equivalent dose of testosterone. This result suggests that testosterone-induced NO release from HCAECs is likely mediated through sAR and acts in a non-genomic manner.

There is evidence that some of the biological actions of testosterone could be mediated through estrogen receptor (ER) via conversion to estrogen by aromatase enzyme.[82,83] We used DHT, a non-aromatizable androgen, to rule out this possibility. An equivalent dose of DHT could enhance the NO release from HCAECs in the same manner and magnitude as testosterone. These results suggest that testosterone's effect on enhancing NO release from HCAECs was mainly mediated through sAR and not through ER, in agreement with the results from other studies suggesting AR involvement in NO regulation.[16,18,19]

Previously, the existence of Ca^{2+}-independent pathway involving c-Src as the upstream regulator and activating eNOS at Ser1177 via either PI3K/Akt or ERK1/2 pathways has been demonstrated.[17-19] There were data suggesting that sAR co-localized with caveolin-1 and was expressed in caveolin-enriched fraction of cell membrane.[18] When testosterone binds to the sAR, sAR, caveolin-1, and c-Src formed a complex leading to increased c-Src phosphorylation at Tyr416, initiation of c-Src/PI3K/Akt cascade, and Akt phosphorylation of eNOS causing eNOS activation in a Ca^{2+}-independent manner.[18] Conversely, ample data have identified Ca^{2+} sources,[57,84-89] binding of Ca^{2+} with CaM, and interaction of Ca^{2+}-CaM complex with calmodulin-binding domain of eNOS facilitating NO production from eNOS,[57,63,64,66,72,84-89] indicating a Ca^{2+}-dependent activation of eNOS.[72] Therefore, to ascertain the role of the Ca^{2+}-dependent pathway in testosterone action, the known Ca^{2+}-independent pathway was blocked at its upstream regulator, c-Src, using PP2.

To our surprise, when T-BSA was given to PP2-pretreated HCAECs, the NO release was significantly higher than T-BSA only group. Previously, NO has been shown to negatively regulate eNOS by inducing c-Src-dependent caveolin-1 phosphorylation which promote eNOS/caveolin-1 interaction resulting in an inhibition of eNOS activity.[90] Therefore, the result we observed could be the outcome of the disinhibition of the negative feedback regulation of eNOS by PP2.

Our lab's previous result that testosterone enhanced HCAEC whole-cell currents by exerting through $G\alpha_{i/o}$/PKA-dependent pathway[12] was not supported in this study: When $G\alpha_{i/o}$/PKA-dependent pathway was blocked using specific blockers for $G\alpha_{i/o}$ and PKA (PTX and H-89, respectively) the testosterone-induced NO release was not attenuated. This discrepancy could be due to the differences in the experimental settings and techniques used. In our study, the cells and intracellular components are intact while in the previous electrophysiological study, using whole-cell patch clamp technique, most of the intracellular components are dialyzed. This may render the $G\alpha_{i/o}$/PKA-dependent pathway to be the main pathway for membrane-related event while it has only minor contribution in intact cells. Our data, however, suggests that $G\alpha_{i/o}$/PKA-dependent pathway is not the only pathway, hence not necessary, for Ca^{2+}-dependent induction of testosterone-induced NO release in intact HCAECs.

Many studies illustrated the involvement of BK_{Ca} and SK_{Ca} channels in the regulation of vascular diameter[91,92] and suggested that testosterone-induced currents in HCAECs mainly passed through these channels.[12] In our study, we used specific blocker for BK_{Ca} and SK_{Ca} channels, IbTX and Apa respectively, to examine the roles of these channels in testosterone-induced NO release. When either BK_{Ca} or SK_{Ca} channels was blocked separately in the presence of PP2, there was no significant effect on testosterone-induced NO release from HCAECs. However, when both BK_{Ca} and SK_{Ca} channels were simultaneously blocked (PP2 still present) testosterone-induced NO release was completely abolished. These data suggest that either BK_{Ca} or SK_{Ca} channels is sufficient for testosterone-induced Ca^{2+}-dependent NO increase, presumably necessary for regulating Ca^{2+} entry.

Nonetheless, simultaneous blockage of BK_{Ca} and SK_{Ca} channels in the absence of PP2, produced only a decline in NO release compared to T-BSA only condition. Previously it has been shown that both BK_{Ca} and SK_{Ca} channels play a significant role in testosterone-induced Ca^{2+}-dependent NO increase. However, when Ca^{2+}-independent pathway was intact, the significant role of BK_{Ca} and SK_{Ca} channels in Ca^{2+}-dependent pathway can be compensated. These data suggest that the Ca^{2+}-independent pathway can compensate for the Ca^{2+}-dependent pathway, consistent with a significant role of the Ca^{2+}-independent pathway described previously.[17-19]

The additional knowledge provided by the present study could further our understanding about the role of testosterone in modulating the circulation and could have useful implications in clinical development of managements and therapies for individuals with cardiovascular risk factors or suffering from cardiovascular diseases.

Limitations: Cells in culture could have a different protein expression profile from those *in vivo*. Data from previous studies suggested that BK_{Ca} channels are absent and do not play any significant functional role in freshly isolated or intact endothelial cells[93,94] while

its expression levels increase with subculturing in cultured cells.[95-101] Therefore, in endothelial cells *in vivo*, SK_{Ca} could have the main role.

Additionally, in this study, c-Src was blocked with PP2. Since NO has been shown to negatively regulate eNOS by acting through a c-Src-dependent mechanism,[90] inhibition of c-Src, the upstream regulator of Ca^{2+}-independent pathway, may also prevent the NO negative feedback of eNOS, causing enhanced NO release. To specifically determine the contribution of Ca^{2+}-independent pathway to the phenomenon while preserving the negative feedback action of NO on eNOS, similar experiments could be further conducted using specific blockers of two known Ca^{2+}-independent pathways, PI3K/Akt and MEK/ERK1/2. Moreover, to ascertain the detailed regulation of BK_{Ca} and SK_{Ca} channels by testosterone, complementary electrophysiological study could also be carried out.

Conclusion

From this study, we can conclude that testosterone, via its androgenic action and acting through sAR, enhances NO release from HCAECs. Moreover, to activate eNOS in Ca^{2+}-dependent manner, both BK_{Ca} and SK_{Ca} play significant roles and both of the channel types can compensate for the activity of each other. The resulted data also suggested that both Ca^{2+}-dependent and -independent pathways may play their own role but Ca^{2+}-independent pathway can compensate for Ca^{2+}-dependent pathway. Whether Ca^{2+}-dependent pathway can compensate Ca^{2+}-independent pathway still need to be verified by using specific blockers of component of Ca^{2+}-independent pathway rather than PP2 as it not only blocked the Ca^{2+}-independent pathway but it also blocked the negative feedback regulation of eNOS by NO in testosterone-induced NO release from HCAECs. The current study is the first study to prove the significant involvement of BK_{Ca} or SK_{Ca} in testosterone-induced NO release from HCAECs by measuring the actual NO release. The information from this study can further the understanding of testosterone action on the cardiovascular system. With further studies, combined information may lead to safer usage of testosterone in clinical setting.

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Conflict of Interest

None to declare.

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