Nontranscriptional Regulation of Cardiac Repolarization Currents by Testosterone

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Background—Women have longer QT intervals than men and are at greater risk for arrhythmias associated with long QT intervals, such as drug-induced torsade de pointes. Recent clinical and experimental data suggest an important role of testosterone in sex-related differences in ventricular repolarization. However, studies on effects of testosterone on ionic currents in cardiac myocytes are limited.

Methods and Results—We examined effects of testosterone on action potential duration (APD) and membrane currents in isolated guinea pig ventricular myocytes using patch-clamp techniques. Testosterone rapidly shortened APD, with an EC50 of 2.1 to 8.7 nmol/L, which is within the limits of physiological testosterone levels in men. APD shortening by testosterone was mainly due to enhancement of slowly activating delayed rectifier K+ currents (I\textsubscript{Ks}) and suppression of L-type Ca\textsuperscript{2+} currents (I\textsubscript{Ca,L}), because testosterone failed to shorten APD in the presence of an I\textsubscript{Ks} inhibitor, chromanol 293B, and an I\textsubscript{Ca,L} inhibitor, nisoldipine. A nitric oxide (NO) scavenger and an inhibitor of NO synthase 3 (NOS3) reversed the effects of testosterone on APD, which suggests that NO released from NOS3 is responsible for the electrophysiological effects of testosterone. Electrophysiological effects of testosterone were reversed by a blocker of testosterone receptors, a c-Src inhibitor, a phosphatidylinositol 3-kinase inhibitor, and an Akt inhibitor. Immunoblot analysis revealed that testosterone induced phosphorylation of Akt and NOS3.

Conclusions—The nontranscriptional regulation of I\textsubscript{Ks} and I\textsubscript{Ca,L} by testosterone is a novel regulatory mechanism of cardiac repolarization that can potentially contribute to the control of QT intervals by androgen. (Circulation. 2005;112:1701-1710.)

Key Words: nitric oxide ■ long-QT syndrome ■ testosterone ■ potassium current ■ ion channels

Sex-related differences have been reported in the propensity for cardiac arrhythmias related to prolonged cardiac repolarization.1-3 Female gender is an independent risk factor for syncope and sudden death in the congenital long-QT syndrome.1-3 Women are more prone to develop torsade de pointes than men in response to QT-prolonging drugs, with 65% to 75% of drug-induced torsade de pointes occurring in women.4-6 Higher susceptibility of females to the congenital long-QT syndrome and drug-induced torsade de pointes is associated with a sex-related difference in ventricular repolarization in the hearts.7-9

Sex differences in cardiac repolarization are influenced by age. At birth, the QT interval is quite similar between men and women.8-10 On arrival of puberty, the duration of QT intervals in boys shortens, which leaves adult women with longer QT intervals than adult men. The QT interval in men then gradually increases until approximately 60 years of age, when QT intervals approach those of women.8-10 As sex hormone level elevates during puberty, the influence of sex hormones on differences in QT intervals between men and women has been documented.8-10 Bidoggia et al.11 in fact, showed that women with virilization exhibit shorter and faster repolarization times than normal women and castrated men. Pham et al.12 showed in male rabbits that a higher serum testosterone level, but not an estradiol level, is associated with shorter action potential duration (APD) and a lower incidence of proarrhythmias by a K+ channel blocker, dofetilide. They also showed in female rabbits that testosterone diminishes the proarrhythmic effects of dofetilide.13 Taken together, these data suggest that testosterone is an important regulator of sex-related differences in ventricular repolarization and the propensity to arrhythmias. However, the mechanisms that underlie the effects of testosterone on ventricular repolarization remain unknown.

Signaling of gonadal steroids such as testosterone and estradiol has traditionally been identified as a transcriptional control of target genes via the binding of complexes of nuclear receptors and ligands to the genomic consensus
sequence in reproductive organs (“transcriptional mechanism”). 14,15 Recently, several biological actions of gonadal steroids that are too rapid to be compatible with transcriptional mechanisms have been identified in nonreproductive organs. 16,17 In cardiac myocytes, testosterone increases expression of the β1-adrenergic receptor, the L-type Ca2+ channels, and the Na+–Ca2+ exchanger in mRNA. 18 On the other hand, testosterone does not change mRNA expression of rat ERG (ether-a-go-go related gene) but increases the current density of the rapidly activating component of the delayed rectifier K+ current (IKr) with changes in channel gating kinetics. 19 Thus, testosterone modulates cardiac repolarization via both a transcriptional and a nontranscriptional mechanism. Testosterone rapidly induces dilatation of blood vessels and positive inotropism of cardiac muscle, which are abolished by pretreatment with a nitric oxide synthase (NOS) inhibitor, N2-nitro-L-arginine methyl ester. These data imply the interaction of the nongenomic action of testosterone and the nitric oxide (NO) system in cardiovascular regulation.16 We recently reported that NO shortens APD by enhancing the slowly activating component of delayed rectifier K+ currents (IKs) and inhibiting L-type Ca2+ currents (ICa,L) 20,21 These findings prompt us to examine whether testosterone also modulates cardiac repolarization via an NO-dependent mechanism. Our data indicate that testosterone shortens APD by modulating both IKs and ICa,L via a nontranscriptional mechanism.

Methods
The investigation was conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Patch Clamp
Single ventricular myocytes were harvested from hearts of adult guinea pigs of either sex (n=36, white Hartrey) as described previously. 22 Action potentials and membrane currents were recorded with the perforated configuration of the patch-clamp technique with an Axopatch 200B amplifier (Axon Instruments). Action potentials were elicited by passing depolarizing current pulses (<2 ms in duration) of suprathreshold intensity with a frequency of 1 Hz. To record ICa,L, a prepulse (100 ms) was applied to ~40 mV from a holding potential (VH) of ~80 mV to inactivate the Na+ channels and the T-type Ca2+ channels, followed by a 200-ms test pulse (VT) to 0 mV at 1 Hz. IKs were elicited by 3.5-second VT to 50 mV from a VH of ~40 mV at 0.1 Hz or by 0.5-second VT at 1 Hz in some experiments. All experiments were done at 36±1°C. Compositions of pipette solutions and bath solutions used are described in the Data Supplement (available at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.104.523217/DC1). Amphotericin B (600 μg/mL; Sigma-Aldrich) was used in pipette solution to achieve patch perforation. The series resistance was 15.7±1.7 MΩ, the capacitance time constant was 2.5±0.3 ms, and the membrane capacitance was 150±13 pF (n=119).

Recordings of Monophasic Action Potentials and Surface ECG in Isolated Langendorff-Perfused Hearts
Recordings of monophasic action potentials (MAPs) and surface ECGs in isolated guinea pig hearts were done as described previously. 23 Retrograde perfusion was maintained at a constant flow (10 to 12 mL/min) with modified Krebs-Henseleit solution containing (in mmol/L) NaCl 119, KCl 4.8, KH2PO4 1.2, MgSO4 1.2, CaCl2 1.0, glucose 10, and NaHCO3 24.9 and equilibrated with 95% O2/5% CO2 (pH 7.4, 37°C). MAP was recorded with a suction electrode, and the ECG was obtained through a contact Ag-AgCl lead on the left ventricle.

Immunoblot Analysis
Immunoblot analyses were performed as described previously (see Data Supplement). 24 Briefly, cell lysates or tissue homogenates that contained 20 μg of protein were applied to SDS/7.5% acrylamide gel, transferred to PVDF membranes, and subjected to immunoblot analysis by incubation with an anti-NOS3 antibody (Zymed), an anti-Akt antibody (Cell Signaling), an anti-phospho-NOS3 antibody (Zymed), or an anti-phospho-Akt antibody (Cell Signaling) followed by incubation with a horseradish peroxidase–conjugated anti-mouse IgG (DAKO Japan Co. Ltd) or an anti-rabbit IgG (DAKO Japan). Proteins were detected with the advance enhanced chemiluminescence system (Amersham Biosciences).

Reagents
Chromanol 293B was supplied by Hoechst. E-4031 was purchased from Eisai Co. Ltd; testosterone and W7 from Wako; nisoldipine, S-methylisothiourea (SMTU), N-acetyl-L-cysteine (LNAC), 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5-trietamethyl-1H-imidazol-1-yl-oxide-3 (carboxy-PTIO), S-methyl-1-thiocitrulline (SMTC), L-N-(1-iminoethylo)mornithine (L-NIO), sodium nitroprusside (SNP), and 2-(4-morpholino)-8-phenyl-1(4H)-benzopyran-4-1 hydrochloride (LY-294,002), and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalinal-1-1 (ODQ) from Sigma-Aldrich; SH-6 from Merck; and nitlamide from TOCRIS. E-4031, nitlamide, W7, SMTU, LNAC, SMTC, L-NIO, and SNP were dissolved in distilled water. Testosterone was prepared as a 5-mmol/L stock solution in ethanol. Chromanol 293B, nisoldipine, carboxy-PTIO, SH-6, LY-294,002, and ODQ were stored as 100-, 10-, 200-, 20-, 60-, and 20-mmol/L stock solutions in DMSO, respectively. They were dissolved in bath solution to achieve a final concentration described in the text. The final concentration of DMSO (0.05% [vol/vol]) or ethanol (0.01% [vol/vol]) was confirmed to have no significant effects on membrane currents.

Data Analysis
All values are presented as mean±SE. Statistical significance among repeated measures in the control experiments, after testosterone, and after washout or addition of various inhibitors was evaluated by repeated-measure nonparametric Friedman test, those in electrophysiological parameters between males and females by unpaired nonparametric Mann-Whitney test, those between control and various drugs by paired nonparametric Wilcoxon test, those against time-dependent changes in current amplitude (time control) by 2-way ANOVA, and others by multiple comparison with Kruskal-Wallis test followed by Dunn’s multiple comparison test. A probability value less than 0.05 was considered significant.

Results
Testosterone Shortened APD in a Dose-Dependent Manner
In ventricular myocytes isolated from female guinea pigs, testosterone shortened APD in a dose-dependent manner (Figure 1A). Figure 1B depicts dose-response curve for APD at 20% repolarization (APD20; Figure 1B, panel a) and that for APD at 90% repolarization (APD90; Figure 1B, panel b). The EC50 value was 5.5±0.3 mmol/L for APD20 (n=5) and 5.8±0.3 mmol/L for APD90 (n=5). After the effects of testosterone (100 mmol/L) on APD had reached a quasi-steady state on 10-minute treatment with testosterone, application of a blocker of testosterone receptors, nitlamide (1 μmol/L), fully reversed APD shortening (Figure 1C), which indicates that testosterone-induced shortening of APD was via testosterone receptors.
Effects of Testosterone on $I_{Ca,L}$, $I_{Kr}$, and $I_{Ks}$

To explore which ion currents are targets of testosterone, we examined effects of testosterone on $I_{Ca,L}$, $I_{Kr}$, and $I_{Ks}$, major ionic currents determining APD in guinea pig ventricles. Testosterone (100 nmol/L) suppressed $I_{Ca,L}$ (Figure 2A) and enhanced $I_{Ks}$ (Figure 2B), whereas it barely affected $I_{Kr}$ (data not shown). Although we recorded $I_{Ks}$ elicited by 3.5-second test pulses at 0.1 Hz to exaggerate the amplitudes unless otherwise noted, we confirmed similar enhancement of $I_{Ks}$ elicited by shorter pulses (0.5 second) at 1 Hz, which is a more physiologically normal condition (Figure 2C). Suppression of $I_{Ca,L}$ and enhancement of $I_{Ks}$ by testosterone were completely inhibited by nilutamide (1 μmol/L; Figure 2). $I_{Ca,L}$ suppression and $I_{Ks}$ enhancement by testosterone were dose-dependent, with a significantly lower EC50 value for enhancement of $I_{Ks}$ than for suppression of $I_{Ca,L}$ (Figure 3).

Next, we examined effects of testosterone on APD and membrane currents in cardiomyocytes isolated from male guinea pigs. In cells from males, baseline parameters and dose-dependent effects of testosterone on APD or membrane currents ($I_{Ca,L}$, $I_{Ks}$) were not significantly different from those in female cells (Data Supplement Tables I and II and Figure I). In the following experiments, therefore, only data obtained from female guinea pig cells are presented.

Relative Contribution of $I_{Ca,L}$ Suppression and $I_{Ks}$ Enhancement to APD Shortening by Testosterone

To examine the relative contribution of $I_{Ca,L}$ suppression and $I_{Ks}$ enhancement to APD shortening by testosterone, we used an $I_{Ca,L}$ inhibitor, nisoldipine, and/or an $I_{Ks}$ inhibitor, chromanol 293B. Nisoldipine is reported not to affect delayed rectifier $K^+$ currents at 10 μmol/L.23 Chromanol 293B is also reported to almost completely inhibit $I_{Ks}$, with minor effects on the transient outward current ($I_{to}$), at 10 μmol/L.24 We confirmed that chromanol 293B at 10 μmol/L barely affected $I_{Ca,L}$ or $I_{Ks}$ in our experimental conditions (Data Supplement Figure II). Cardiac myocytes were preincubated with nisoldipine (3 μmol/L) and/or chromanol 293B (10 μmol/L), and subsequently, the effects of testosterone were examined (Figure 4; Data Supplement Figure III). For testosterone at 1 nmol/L, preincubation with nisoldipine did not affect the magnitude of APD shortening, whereas preincubation with chromanol 293B alone or chromanol 293B plus nisoldipine abolished APD shortening by testosterone (Figures 4A and 4C). For testosterone at 100 nmol/L, preincubation with nisoldipine diminished APD shortening by 37.2±1.1% for APD20 and 38.6±2.2% for APD90, and preincubation with chromanol 293B diminished it by 59.8±1.5% for APD20 and 56.7±1.4% for APD90. Both blockers together almost completely abolished APD shortening by testosterone (89.2±0.8% for APD20 and 90.1±1.4% for APD90; Figures 4B and 4C). These data suggest that testosterone-dependent modulation of $I_{Ks}$ and $I_{Ca,L}$ accounts for APD shortening induced by testosterone; at a low concentration of testosterone, APD shortening is mainly due to enhancement of $I_{Ks}$, whereas at a high concentration, both $I_{Ks}$ enhancement and $I_{Ca,L}$ suppression contribute to APD shortening.

Effects of Testosterone Are Mediated by Actions of NO

We have previously reported that NO shortens APD by suppressing $I_{Ca,L}$ and enhancing $I_{Ks}$.21 as does testosterone. We hypothesized that the electrophysiological effects of testosterone are mediated by NO actions. After APD shortening by testosterone (100 nmol/L) had reached a quasi-steady state, it was completely reversed by an NOS inhibitor, SMTU (1 μmol/L; Figure 5A), and by the NO scavengers LNAC (1 mmol/L; Figure 5B) and carboxy-PTIO (100 μmol/L; Data Supplement Figure IV). Similarly, $I_{Ca,L}$ suppression and $I_{Ks}$ enhancement by testosterone were reversed by SMTU (1 μmol/L; Figures 5C and 5E), LNAC (1 mmol/L; Figures 5D and 5F), and carboxy-PTIO (100 μmol/L; Data Supplement Figure VI). These data suggest that the effects of testosterone on APD, $I_{Ca,L}$, and $I_{Kr}$ are mediated by an increase in cytosolic NO.
Modulation of \( I_{Ca,L} \) and \( I_{Ks} \) Is Mediated by Akt-Dependent Activation of NOS3

Amplitudes of \( I_{Ca,L} \) and \( I_{Ks} \) were increased rapidly (within \( \approx 5 \) minutes) after application of testosterone, which argues against activation of inducible NOS (NOS2). Therefore, we tested whether testosterone activated NOS1 or NOS3 to affect \( I_{Ca,L} \) and \( I_{Ks} \). We used 2 different NOS inhibitors, SMTC, which is more sensitive to NOS1 (IC\(_{50}\) = 0.31 \( \mu \)mol/L) than to NOS3 (IC\(_{50}\) = 5.4 \( \mu \)mol/L), and L-NIO, which is more sensitive to NOS3 (IC\(_{50}\) = 0.5 \( \mu \)mol/L) than to NOS1 (IC\(_{50}\) = 3.9 \( \mu \)mol/L). Application of SMTC (3 \( \mu \)mol/L) did not alter suppression of \( I_{Ca,L} \) by testosterone, whereas L-NIO (1 \( \mu \)mol/L) reversed testosterone-induced suppression of \( I_{Ca,L} \) to its initial levels (Figure 6A). Similarly, SMTC (3 \( \mu \)mol/L) did not alter enhancement of \( I_{Ks} \) by testosterone, whereas L-NIO (1 \( \mu \)mol/L) reversed the testosterone-induced enhancement of \( I_{Ks} \) to its initial levels (Figure 6B). These findings suggest that the effects of testosterone on \( I_{Ca,L} \) and \( I_{Ks} \) are mediated by NO released from NOS3.

NOS3 is activated via at least 2 distinct pathways, a phosphorylation-dependent pathway involving the Ser/Thr kinase, Akt, or a \( Ca^{2+} \)-dependent pathway involving the \( Ca^{2+} \)-binding protein, calmodulin (CaM). To test which pathway is involved in testosterone-induced NOS3 activation, we used an Akt inhibitor (SH-6) and a CaM inhibitor (W7). SH-6 (10 \( \mu \)mol/L) reversed suppression of \( I_{Ca,L} \) and enhancement of \( I_{Ks} \) by testosterone back to the initial levels, whereas W7 (5 \( \mu \)mol/L) did not alter either \( I_{Ca,L} \) suppression or \( I_{Ks} \) enhancement (Figures 6A and 6B). Testosterone activates NOS3 through its nongenomic (nonnuclear) pathway, in which binding of testosterone to membrane-localized testosterone receptors activates tyrosine kinase, c-Src, followed by sequential activation of phosphatidylinositol 3-kinase (PI-3 kinase), Akt, and then NOS3. Therefore, we further tested involvement of c-Src and PI-3 kinase in testosterone-induced effects on \( I_{Ca,L} \) and \( I_{Ks} \) using a c-Src inhibitor, PP2, and a PI-3 kinase inhibitor, LY-294,002. Both PP2 (10 \( \mu \)mol/L) and LY-294,002 (30 \( \mu \)mol/L) reversed suppression of \( I_{Ca,L} \) and enhancement of \( I_{Ks} \) by testosterone back to their initial levels (Figures 6A and 6B).

Effects of Testosterone on Phosphorylation of Akt and NOS3

Akt is phosphorylated at \( ^{473} \)Ser on its activation. Full activation of NOS3 is achieved by phosphorylation of \( ^{1177} \)Ser by Akt. We therefore investigated whether testosterone induced...
phosphorylation of Akt at Ser177 and NOS3 at Ser1177 by immunoblot analysis using antibodies against phospho-Akt and phospho-NOS3. Incubation of cardiac myocytes with testosterone for 10 minutes increased phosphorylation of both Akt and NOS3 in a concentration-dependent manner (Figure 7A). Phosphorylation of Akt and NOS3 was inhibited by nilutamide, PP2, LY-294,002, and SH-6 but not by W7 (Figure 7B). These immunoblot results were consistent with our electrophysiological data.

Effects of Testosterone on MAPs and QT Interval in Isolated Hearts

Effects of testosterone in isolated whole hearts were examined in Langendorff-perfused hearts. Perfusion of testosterone one reversibly shortened duration of MAPs at 20% repolarization and at 90% repolarization and shortened the QTc interval within 15 minutes (Figures 8A and 8B).

Left ventricles were homogenated after the same amount of time (15 minutes) of perfusion with or without testosterone as functional experiments, and immunoblot analyses were performed. Immunodensity of phospho-Akt and phospho-NOS3 was higher in hearts with testosterone perfusion than in nontreated hearts (Figure 8C).

Discussion

Although recent clinical and basic studies suggest important regulatory roles of testosterone in cardiac repolarization,8–13
underlying mechanisms remain unknown. We have demonstrated the following in the present study: (1) Testosterone shortens APD at an EC50 of 4.5 ± 0.6 nmol/L for APD20 and 2.7 ± 0.6 nmol/L for APD90, which is within the physiological range of serum testosterone levels in men. Serum testosterone level is reported to be 10.4 to 34.7 nmol/L in men and 0.6 to 2.7 nmol/L in women. (2) Testosterone-induced APD shortening is mainly due to enhancement of $I_{Ks}$, in part with a contribution of $I_{Ca,L}$ suppression at a high concentration of testosterone. (3) $I_{Ks}$ enhancement and $I_{Ca,L}$ suppression are due to NOS3 activation and NO production through a nongenomic pathway, in which c-Src, PI-3 kinase, Akt, and NOS3 are sequentially activated.

**Effects of Testosterone on Cardiac Ion Currents**

In the present study, APD shortening by testosterone up to 100 nmol/L was fully abolished by preincubation with chromanol 293B and nisoldipine, which suggests that testosterone-induced APD shortening was mainly caused by its effects on $I_{Ks}$ and $I_{Ca,L}$ in guinea pig ventricular myocytes. There are several studies investigating the effects of androgens on cardiac currents. Epiandrosterone is a metabolite of the testosterone precursor dehydroepiandrosterone. Gupte et al. demonstrated that epiandrosterone suppressed $I_{Ca,L}$ in rat ventricular myocytes. Pham et al. have suggested involvement of the $I_{Ks}$ component in the effects of testosterone. Shuba et al. reported in the Xenopus laevis heterologous expression system that testosterone produced a 35% reduction in expressed human ERG (HERG) currents. The concentration of testosterone they used (1 μmol/L) was beyond the range of its serum concentration in normal male mammals, including humans. Chronic application of androgen in vivo exhibited enhancement of the inward rectifier K+ current ($I_{K1}$) and $I_{Ks}$ in rabbit. In the present study, we found no apparent effects of testosterone on $I_{Ks}$ at concentrations up to 100 nmol/L. Although we have no clear explanation for the difference, it may reflect different modes of testosterone application (chronic versus acute) or species differences.

**Signaling Pathway of Testosterone-Dependent Current Modulation**

In cardiac myocytes, testosterone has been shown to exhibit acute effects independently of a transcriptional mechanism, in addition to the conventional genotropic action. Androgen receptors have no intrinsic transmem-
Effects of these inhibitors were via interference with the plasma membrane domain, and it has not been clarified how androgen receptors are localized to the plasma membrane. Although interaction with a scaffolding protein, caveolin-1, is postulated to act for membrane localization of androgen receptors,36 no direct interaction between testosterone receptors and heart-type caveolin (caveolin-3) has been determined. Nevertheless, binding of testosterone to receptors on the plasma membrane is suggested to sequentially activate c-Src, PI-3 kinase, and Akt.31,17 In the present study, each inhibitor for testosterone receptors, c-Src, PI3-kinase, Akt, and NOS3, reversed testosterone-induced $I_{Ks}$ enhancement and $I_{Ca,L}$ suppression. To confirm that effects of these inhibitors were via interference with

effects of testosterone, we performed additional control experiments: in the absence of testosterone, application of nitulamide, PP2, LY-294,002, SH-6, and L-NIO affected neither $I_{Ks}$ nor $I_{Ca,L}$, and subsequent application of testosterone did not exhibit $I_{Ks}$ enhancement or $I_{Ca,L}$ suppression (Data Supplement Figures VII, VIII, and IX). Testosterone treatment induced phosphorylation of both Akt and NOS3, which were inhibited by these reagents. Thus, the present study provides evidence of testosterone regulation of cardiac ion channels through a nontranscriptional mechanism (Figure 9).

**Differential Dose Dependence of Testosterone on $I_{Ca,L}$ and $I_{Ks}$**

Testosterone-induced APD shortening is mainly caused by enhancement of $I_{Ks}$ at a low concentration (1 nmol/L), whereas at a high concentration of testosterone, it is caused by the effects of testosterone on both $I_{Ks}$ and $I_{Ca,L}$. These data are consistent with the findings that testosterone enhanced $I_{Ks}$ with an EC$_{50}$ of 1.1±0.2 nmol/L, whereas testosterone required a relatively higher concentration to suppress $I_{Ca,L}$ (IC$_{50}$=38.8±3.5 nmol/L).

Although suppression of $I_{Ca,L}$ and enhancement of $I_{Ks}$ by testosterone are mediated through the same pathway from activation of c-Src to NOS3 activation, the concentration of testosterone to modulate $I_{Ca,L}$ and $I_{Ks}$ differ. This could be explained by differential mechanisms of NO to regulate $I_{Ca,L}$ and $I_{Ks}$. In our previous report, suppression of $I_{Ca,L}$ by NO was dependent on cGMP, which suggests that phosphorylation by protein kinase G is involved.20 By contrast, enhancement of $I_{Ks}$ by NO is not dependent on cGMP.20 In the present study, effects of testosterone on $I_{Ca,L}$, but not on $I_{Ks}$, were also inhibited by a guanylate cyclase inhibitor, ODQ (Data Supplement Figure X). Furthermore, the concentration of an NO donor, SNP, required to modulate $I_{Ca,L}$ was significantly higher than that required to modulate $I_{Ks}$, (Data Supplement Figure XI), which suggests that sensitivity of NO to different between $I_{Ca,L}$ and $I_{Ks}$. However, direct measurement of the sensitivity of NO on guanylate cyclase and protein S-nitrosylation has not been addressed.

**Clinical Implications and Study Limitations**

Virilized women have shorter JT intervals than castrated men. Men have longer JT intervals after orchietomy.11 In men, QT intervals shorten at puberty.9,10 Furthermore, there is a tendency toward an age-dependent reduction in the number of male patients with long-QT syndrome who manifest QT, intervals >440 ms.38 These clinical findings implicate a unique modulatory role of testosterone in ventricular repolarization. However, it is currently unproved whether the nontranscriptional regulation of cardiac repolarization currents by testosterone demonstrated in the present study underlies sex-related differences in QT interval in humans. Although we demonstrated that testosterone shortens APD and QTc interval in isolated intact hearts, the contribution of this regulatory mechanism in the physiological condition remains unknown. Because testosterone is suggested to modulate cardiac repolarization via both a transcriptional and a nontranscriptional mechanism in cardiac myocytes,18,19,39 transcriptional regulation by testosterone should be tested in

**Figure 6. Effects of inhibitors of the signal molecules in the nongenomic pathway of testosterone.** A. Effects of various inhibitors on $I_{Ca,L}$ suppression by testosterone (100 nmol/L). The percent reversal of $I_{Ca,L}$ suppression by various inhibitors was calculated as 100×[$I_{Ca,L}$(testosterone)−$I_{Ca,L}$(inhibitors)]/[−$I_{Ca,L}$(testosterone)−$I_{Ca,L}$(control)] with 5 experiments. *P<0.05 vs control. B. Effects of various inhibitors on $I_{Ks}$ enhancement by testosterone (100 nmol/L). The percent reversal of $I_{Ks}$ enhancement by various inhibitors was calculated as 100×[$I_{Ks}$(testosterone)−$I_{Ks}$(inhibitors)]/[−$I_{Ks}$(testosterone)−$I_{Ks}$(control)] with 5 experiments. *P<0.05 vs control.
addition to nontranscriptional regulation to investigate its contribution in physiological conditions. In the present study, we used guinea pig hearts that lack $I_{Ca,L}$ to, which is different from human hearts. Thus, the contribution of $I_{Ks}$ to sex differences in cardiac repolarization has not been addressed in the present study. Estradiol also acutely activates NOS3 independently of genotropic action.40,41 In fact, in preliminary experiments, we found that estradiol modulates $I_{Ca,L}$ and $I_{Ks}$ via NOS3 activation (Data Supplement Figure XII). However, our data suggested the presence of crucial qualitative and quantitative differences in electrophysiological effects between testosterone and estradiol (Data Supplement Figure XII). Further studies are certainly required to clarify the role of nontranscriptional regulation of testosterone on the gender-dependent difference in the QTc interval and propensity to arrhythmias.

Figure 7. Phosphorylation of Akt and NOS3 by testosterone. A, Dose-dependent phosphorylation of Akt or NOS3 by testosterone. Left panel depicts immunoblot (IB) analysis by anti-phospho-Akt (first panel), anti-Akt (second panel), anti-phospho-NOS3 (third panel), or anti-NOS3 (bottom panel) antibody in the absence or presence of testosterone at various concentrations. The right panel shows densitometric analysis averaged from 3 experiments. *$P<0.05$ vs control for Akt; †$P<0.05$ vs control for NOS3. B, Effects of various blockers on phosphorylation of Akt or NOS3. Left panel depicts immunoblot analysis in the control state (lane 1), in the presence of 100 nmol/L testosterone alone (lane 2), or in the presence of 100 nmol/L testosterone and each of various inhibitors (lanes 3 to 7). Right panel shows densitometric analysis averaged from 3 experiments. *$P<0.05$ vs control for Akt; †$P<0.05$ vs control for NOS3.

Figure 8. Effects of testosterone on isolated Langendorff-perfused hearts. A, Effects of retrograde perfusion of coronary artery with solution containing testosterone (100 nmol/L) on APD$_{20}$ and APD$_{90}$ of MAP and QTc interval. *$P<0.05$ vs control; †$P<0.05$ vs testosterone. B, Representative recordings of MAP (left panel) and ventricular ECG in the control state (a), in the presence of testosterone (100 nmol/L; b), and during washout (c). Vertical lines represent the beginning of MAP or QRS wave, 90% repolarization of MAP, and the end of T wave. C, Phosphorylation of Akt and NOS3 by testosterone (100 nmol/L) in isolated hearts. Right panel shows densitometric analysis averaged from 3 experiments. IB indicates immunoblot. *$P<0.05$ between control and testosterone.
Figure 9. Proposed schematic model for regulatory mechanism of testosterone on \(I_{Ca,L}\) and \(I_{Ks}\). A, In the ligand-free condition, NOS3 is interacted with membrane-associated caveolin-3 and is in an inactive state. The rapid effect of testosterone on \(I_{Ca,L}\) and \(I_{Ks}\) suggests an involvement of testosterone receptor on the plasma membrane, although molecular identity and mechanism of plasma membrane localization for testosterone receptor have not yet been clarified. B, When testosterone binds to its receptors, the testosterone receptor, c-Src, and \(p85\) of PI3-kinase form a macromolecular complex, which renders \(p110\) of PI3-kinase free and in an active state, and active PI3-kinase converts PIP(3,4,5) into PIP3(3,4,5). Subsequently, PIP3(3,4,5), Akt, and NOS3 form a complex, which results in NOS3 activation and NO production. Produced NO then inhibits \(I_{Ca,L}\) via a cGMP-dependent manner and enhances \(I_{Ks}\) via a cGMP-independent manner.

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References


**CLINICAL PERSPECTIVE**

Polymorphic ventricular tachycardias associated with QT prolongation, known as torsade de pointes, occur as a manifestation of the congenital long-QT syndrome or can be acquired from any agent that induces QT prolongation. Gender influences the phenotypic manifestations of congenital long-QT syndrome and the susceptibility to acquired long-QT syndrome, with women known to be at greater risk. Torsade de pointes occurs with similar frequency in boys and girls with congenital long-QT syndrome until puberty. After puberty, it is less common in boys, whereas women have a greater risk. This observation fits with the gender difference in QT interval observed in the general population. The QT interval is similar between boys and girls until puberty, when it shortens only in men and then remains shorter in men than in women until around the age of 50 years. Because this period of life coincides with the highest circulating levels of testosterone in men, it seems plausible that testosterone modulates the QT interval. In the present study, we demonstrated that testosterone applied to isolated hearts or single cardiac myocytes rapidly shortened QT interval and APD. Effects of gonadal steroids occur either via transcription of genes with hormone-responsive elements or by mechanisms that do not require gene transcription (“nongenomic action”). Testosterone-induced modulation of cardiac repolarization currents found in the present study is attributable to a nongenomic action, in which nitric oxide produced by an Akt/endothelial nitric oxide synthase–dependent pathway is a main effector. In addition to the genomic regulation of ion channel expression by testosterone reported elsewhere, this novel regulatory mechanism of cardiac ion currents by testosterone may shorten the QT interval in men during the reproductive age, providing a potential mechanism of gender differences in QT intervals and torsade de pointes risk.
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