Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel

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Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel. Moreover, inhibiting BKCa channels in single coronary myocytes, and further analysis identified this protein as the large-conductance, calcium-activated potassium channel. Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel.

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THE INCIDENCE OF CARDIOVASCULAR disease is influenced by both gender and age. For example, the risk of developing coronary artery disease or hypertension is much higher in men than in premenopausal women; however, by the age of 65 years a woman is just as likely to suffer cardiovascular dysfunction as a man of similar age (12, 14). Therefore, it has been proposed that gonadal steroids influence cardiovascular physiology and/or pathophysiology. Specifically, it has been proposed that estrogen may protect against the development of cardiovascular diseases; however, the administration of exogenous estrogen in men may actually increase the risk of death from coronary artery disease (1). Testosterone, on the other hand, is often considered to exacerbate the development of cardiovascular diseases; however, clinical and epidemiological studies of the relationship between testosterone and cardiovascular disease are at best controversial. For example, plasma testosterone levels are reported to correlate either positively or negatively with the incidence of coronary artery disease in men (3, 17). In fact, testosterone is associated with higher levels of high-density lipoprotein in men and was correlated negatively with risk factors such as fibrinogen, plasminogen activator inhibitor-1, and insulin (16), suggesting that hypotestosteronemia may be a risk factor for coronary atherosclerotic heart disease in men. In addition, plasma androgen levels are higher in normotensive males than in their hypertensive counterparts (12).

Interestingly, potential therapeutic effects of testosterone on angina pectoris were first reported over 50 years ago (10, 21), with more recent electrocardiographic studies demonstrating that testosterone relieves exercise-induced S-T segment depression (11). In light of these studies, it seems premature to conclude that testosterone promotes cardiovascular dysfunction. Instead, a better understanding of the cellular and molecular effects of testosterone on the cardiovascular system is needed before any definitive conclusions can be made regarding the role of testosterone in cardiovascular disease.

Recent in vitro studies revealed that testosterone produces acute (within minutes) endothelium-independent relaxation of rabbit coronary arteries (24). More...
recent studies from our laboratory demonstrated that testosterone-induced relaxation of the rat aorta is gender and androgen receptor independent and involves both endothelium-dependent and -independent mechanisms (4). Endothelium-dependent and endothelium-independent vasodilatory effects of testosterone were also described in canine coronary conductance and resistance arteries in vivo (2). Interestingly, pharmacological evidence from each of these previous studies suggests that testosterone-induced vascular relaxation might involve potassium efflux; however, to our knowledge, no studies have yet investigated potential effects of testosterone on potassium channels directly with the patch-clamp technique nor has an effect of testosterone on porcine coronary arteries been reported. The purpose of the present study was to assess the direct effect of testosterone on porcine coronary arteries and single myocytes from these vessels. Using isometric coronary vascular preparations and single-channel, patch-clamp recordings, we have identified a specific large-conductance, calcium- and voltage-activated potassium (BKCa) channel as the primary effector mediating testosterone-induced relaxation of porcine coronary arteries.

METHODS

Arterial tension studies. Fresh porcine hearts from castrated males or gilts were obtained from local abattoirs. The left anterior descending (LAD) artery was excised and placed in ice-cold, low-calcium dissociation medium (DM) of the following composition (in mM): 110 NaCl, 5 KCl, 0.16 CaCl2, 2 MgCl2, 10 HEPES, 10 NaHCO3, 0.5 Na2HPO4, 10 glucose, 0.49 EDTA, and 10 taurine (pH 6.9). The arteries were kept on ice during their transport to the laboratory. Arterial rings (length, 2 to 4 mm) were prepared from the LADs and mounted in organ baths for isometric tension recording using standard methods. The endothelium was removed in some rings by passing a fray nylon string through the vessel lumen and gently rubbing the intimal surface. The arterial rings were suspended in organ baths containing Krebs-Henseleit bicarbonate (KHB) solution (37°C, gassed with 95% O2-5% CO2) of the following composition (in mM): 110 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 NaHCO3, 1.2 MgCl2, 1.2 KH2PO4, and 11 glucose (pH 7.4). Preparations were equilibrated for 90 min at an optimal passive tension of 2.5 g. Fresh KHB solution was added every 20 min. After equilibration, rings were stabilized by two successive exposures to the cytoplasmic surface of the membrane consistent of the following low-calcium solution (in mM): 60 K2SO4, 30 KCl, 2 MgCl2, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, 0.16 CaCl2 (pCa 7), 10 HEPES, 5 ATP, and 10 glucose (pH 7.4; 22–25°C).

Biochemical analysis. cGMP was measured as described previously (23) by using an enzyme immunometric assay kit (Biomol) that included all reagents, antibodies, and microtiter plates. Briefly, endothelium-denuded media strips from coronary arteries were exposed to a single concentration of either 10 μM or 50 μM testosterone or 10 μM sodium nitroprusside (as a positive control) for 30 min with 10 μM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity. Reactions were stopped by adding 0.1 N HCl and boiling for 5 min. The precipitated protein was removed by centrifugation. After colorimetric analysis, nucleotide levels were expressed as femtomoles of nucleotide per milligram tissue weight.

Statistical analysis. Statistical significance between two groups was evaluated by Student’s t-test for paired data. Comparison among multiple groups were made using a one-way ANOVA test, followed by Tukey’s test post hoc to determine significant differences among the means of the data groups. A probability of P < 0.05 was accepted as a significant difference. For functional studies, n = number of porcine hearts employed in the study; for patch-clamp studies, n = number of patches studied.

Drugs. 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid was purchased from Calbiochem. All other agents were purchased from Sigma.
RESULTS

Arterial tension studies. Testosterone produced concentration-dependent relaxation of coronary arterial ring preparations precontracted with PGF$_{2\alpha}$. A complete concentration-response relationship for testosterone-induced relaxation of intact arteries is illustrated in Fig. 1, which reveals that testosterone (75 μM) induces a nearly complete relaxation of 97.4 ± 1% ($n = 6$). In contrast, precontracted preparations exposed to ethanol (vehicle control) or PGF$_{2\alpha}$ alone (time control) relaxed no more than an average of 11.3 ± 2%. The sensitivity (EC$_{50}$ value) of coronary arterial rings to testosterone-induced relaxation was 26.4 ± 4 μM. The importance of endothelium in mediating testosterone-induced coronary arteries relaxation was investigated by obtaining a series of complete concentration-response relationships (Fig. 2). In these experiments, removal of the endothelium resulted in a slight but insignificant shift in the testosterone response curve (EC$_{50}$ values: 31.3 ± 4.8 μM, intact arteries; 44.4 ± 9.7 μM, endothelium removed; $n = 7$; $P > 0.05$). A similar effect was observed when intact arteries were precontracted for 30 min with 250 μM N$^\omega$-nitro-L-arginine methyl ester (EC$_{50}$ value, 39.3 ± 3.5 μM; $n = 7$ arteries), an inhibitor of nitric oxide synthesis. Furthermore, the maximal relaxation response was similar under all conditions tested (~85%, $n = 28$ arteries). Because these findings are consistent with previous studies indicating that testosterone induces endothelium-independent relaxation of coronary arteries (24), subsequent tension studies employed endothelium-denuded coronary rings to control for potential indirect effects of vasoactive factors released from endothelium.

Because testosterone can be converted into estrogen by cellular aromatase activity and we had previously reported that estrogen relaxes porcine coronary arteries (22), it was possible that the relaxing effect of testosterone on these arteries might be indirectly mediated by conversion to estrogen. Dihydrotestosterone (DHT), a nonaromatizable testosterone metabolite, was employed to test this possibility. DHT (100 μM) induced a 36.9 ± 3.42% ($n = 6$) relaxation of coronary arteries precontracted with 10 μM PGF$_{2\alpha}$ (Fig. 3) and exhibited a time course similar to that of testosterone-induced relaxation (Fig. 4).

In solutions with 5 mM physiological concentration of extracellular K$^+$ ([K$^+$]o), endothelium-denuded arteries precontracted with 10 μM PGF$_{2\alpha}$ relaxed 77.2 ± 4.1% ($n = 8$ arteries) in response to a single exposure to 25 μM testosterone (Fig. 4A). In contrast, this same concentration of testosterone produced an average relaxation of only 5.1 ± 1.4% in the same artery precon-
tractions with 80 mM KCl (n = 6). These findings indicate that the majority (~94%) of testosterone-induced coronary relaxation requires potassium gradients suitable for K\(^+\) efflux and further suggested potential involvement of K\(^+\) channels. Furthermore, pretreating coronary arteries with 1–2 mM tetraethylammonium (TEA) induced a contraction relatively resistant to testosterone (Fig. 4B): 25 μM testosterone (with 5 mM [K\(^+\)]\(_o\)) produced only 8.2 ± 1.1% relaxation (n = 6) of TEA-contracted arteries, a response similar to the blunted effect observed in arteries precontracted with 80 mM KCl. At these low concentrations, TEA is a selective inhibitor of BK\(\text{Ca}\) channels. Moreover, testosterone produced only 12.6 ± 3.3% relaxation (n = 6) in arteries precontracted with PGF\(_{2\alpha}\), in the presence of 20 nM iberiotoxin (25 min), a highly selective inhibitor of BK\(\text{Ca}\) channels (Fig. 5). These studies on intact arteries strongly suggested that testosterone induced coronary relaxation by opening BK\(\text{Ca}\) channels in coronary smooth muscle; however, direct evidence for ion channel involvement cannot be obtained from studies on intact tissues. To test the hypothesis that testosterone opened K\(^+\) channels, patch-clamp experiments were performed on isolated coronary myocytes to measure the activity of single K\(^+\) channels directly.

**Patch-clamp studies.** Conclusive evidence for testosterone-induced stimulation of BK\(\text{Ca}\) channel activity was obtained from patch-clamp experiments on isolated coronary myocytes in which the activity of single K\(^+\) channels was measured directly. Recordings from excised inside-out patches demonstrated that membrane electrical activity was dominated by a single species of high-amplitude channel carrying outward current. Biophysical analysis of single-channel, current-voltage relationships revealed a microscopic conductance of 221 ± 11 pS (n = 3–4 studies) in symmetrical K\(^+\) gradients (140 mM; Fig. 6A). In addition, channels were opened by increasing Ca\(^{2+}\) concentration at the cytoplasmic surface of inside-out patches (1 μM; NP\(_o\) 0.39 ± 0.05; n = 4), whereas 1 mM TEA blocked calcium-stimulated channel activity (NP\(_o\) 0.000; Fig. 6B; n = 4). These findings identify this protein as the BK\(\text{Ca}\) channel, which other studies have
The present study is the first to report testosterone-induced relaxation of porcine coronary arteries in vitro. DHT, a nonaromatizable testosterone metabolite, also relaxed coronary arteries, suggesting that aromatization to estrogen is not required to produce this relaxation response. Furthermore, this response required physiological gradients of potassium, suggesting potential involvement of potassium channels. Subsequent patch-clamp studies provided direct molecular evidence that testosterone stimulates the activity of BKCa channels in single coronary myocytes, possibly via cGMP. Moreover, these cellular studies are completely consistent with functional studies of coronary arteries demonstrating that BKCa channels mediate relaxation.
nearly all of testosterone-induced relaxation. Iberiotoxin, a highly specific antagonist of BKCa channels, attenuated testosterone-induced coronary relaxation by 84% (Fig. 5). Therefore, we propose that stimulation of BKCa channel activity can account for the majority of testosterone-induced relaxation of porcine coronary arteries.

Recent studies demonstrated that testosterone relaxes rabbit coronary arteries or aorta (24) and rat thoracic aorta (4) in vitro and canine coronary arteries in vivo (2). Both endothelium-dependent and -independent effects of testosterone are reported in these studies. Therefore, testosterone may have multiple sites of action. In the present study on porcine coronary arteries, removal of the endothelium did not affect testosterone-induced coronary relaxation significantly; therefore, it is highly likely that the primary site of
testosterone action in porcine coronary arteries is the vascular smooth muscle cell. Regardless of the target, it is clear that testosterone modulates the excitability of vascular smooth muscle, and the present study now provides direct molecular evidence that testosterone opens potassium channels in vascular smooth muscle cells. Furthermore, we have identified the BKCa channel as the primary effector molecule mediating this potassium efflux and subsequent relaxation of porcine coronary arteries. Myocytes from both human (9) and porcine (19) coronary arteries express BKCa channels at high density, and because of their large conductance, these channels help set and maintain the resting potential of vascular smooth muscle cells under physiological conditions (20). Moreover, inhibition of BKCa channels by TEA (Fig. 4B) or iberiotoxin (22) induces contraction of porcine coronary arteries in vitro, confirming the importance of these channels in regulating tension under either stimulated or unstimulated conditions. An additional feature of interest regarding these channels is their ability to provide a repolarizing negative-feedback mechanism to reverse active contraction due to increased intracellular levels of calcium. Because single-channel studies clearly demonstrated increased BKCa channel activity to be the predominant effect of testosterone, we conclude that the BKCa channel is an important effector of testosterone in these myocytes. Previous in vitro studies reported that glibenclamide, an inhibitor of the ATP-sensitive potassium channel, had no effect on testosterone-induced relaxation of rabbit coronary arteries (24), although a subsequent study reported that this compound reduced the effect of testosterone on smaller resistance coronary vessels in the dog (2). In addition, neither glibenclamide nor 1 mM TEA inhibited testosterone-induced relaxation of the rat aorta, whereas 4-aminopyridine attenuated the response to testosterone by 44% (7). Taken together, these findings suggest that the nature of potassium channel stimulation by testosterone may be heterogeneous with respect to artery and/or species. However, the identity of K+ channel(s) stimulated in these arteries will remain somewhat speculative until patch-clamp studies are performed on myocytes isolated from each vessel. In contrast, the present study now provides direct evidence that BKCa channel activity is stimulated by testosterone in porcine coronary arteries. In support of our findings, a recent study by Crews and Khalil (5) has demonstrated that testosterone inhibits 45Ca2+ influx in porcine coronary arteries but does not affect release of intracellular calcium. These findings are consistent with those of the present study that strongly suggest that testosterone inhibits calcium channel activity by opening BKCa channels, resulting in hyperpolarization of the vascular cell membranes and closing of the voltage-dependent calcium channels.

Although the present studies have identified an effector molecule (BKCa channel) that mediates testosterone-induced relaxation of coronary arteries, the complete transduction mechanism involved in this process remains to be elucidated. One signaling molecule
in this process appears to be cGMP, which is increased in coronary smooth muscle after treatment with testosterone (Fig. 8B). Furthermore, studies on cell-attached patches verified that cGMP also opened BK_Ca channels in single coronary myocytes, thus mimicking the effect of testosterone on these cells. Therefore, evidence from both functional and biochemical studies is consistent with the hypothesis that cGMP mediates the effect of testosterone on porcine coronary arteries. However, the present study cannot exclude involvement of other potential signaling mechanism. Although it is unclear at present how androgens might stimulate production of cGMP in vascular smooth muscle in the absence of endothelium, a similar non-genomic, nucleotide-dependent mechanism of action has been proposed for other gonadal steroids. For example, estrogen also increases cGMP accumulation and stimulates BK_Ca channel activity in coronary smooth muscle (6).

Because testosterone and estrogen produce similar effects in coronary smooth muscle cells, it was possible that the stimulatory effects of testosterone were actually indirect, i.e., due to aromatization to estrogen. However, the present study suggests that testosterone-induced relaxation of porcine coronary arteries probably involves a direct effect of the androgen molecule on the vasculature. A nonaromatizable metabolite of testosterone, DHT, produced a similar vasodilatory effect in a similar time frame, albeit with an apparently lower sensitivity. This finding is consistent with previous studies of the rat aorta (8) demonstrating that testosterone-induced relaxation was a structurally-specific effect of the androgen molecule. In that study, maximal relaxation by DHT (69%) was substantially less than that produced by testosterone (100%). Furthermore, previous studies have demonstrated that inhibition of aromatase activity with aminoglutethimide had no effect on testosterone-induced relaxation of rabbit coronary arteries (24). Therefore, the present results are consistent with previous findings obtained in other arteries and suggest a direct vasodilatory effect of testosterone that is not likely to depend on conversion to estrogen or other vasoactive steroids. Although it is possible that testosterone is converted to DHT in the vessel wall, this seems unlikely because in virtually all other nonreproductive target tissues, the biological actions of testosterone do not require conversion to DHT. Furthermore, if conversion of testosterone to DHT occurred to any significant extent, then the vasodilatory efficacy and potency of these two androgens should be the same, but evidence from three other androgen analog studies establishes that this is definitely not the case (8, 13, 24). Previous studies have also established that testosterone-induced vascular relaxation is independent of the classical androgen receptor (4). In fact, testosterone conjugated with bovine serum albumin produced a greater relaxation of the rat aorta compared with unconjugated testosterone (8), implicating involvement of a peripheral cell membrane (nonnuclear) site of action. In addition, previous studies of the rabbit coronary artery reported that an-

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pendent relaxation of this vessel, and results from both tissue and single-cell experiments demonstrate that this response primarily involves stimulation of BKCa channel activity. Understanding the signaling mechanisms that couple testosterone receptor activation to K+ channel stimulation will provide a better understanding of the cellular processes underlying the vasorelaxant effects of testosterone. Such future studies will further underscore the importance of steroid hormones in regulating cardiovascular function and also in treating and/or preventing diseases of the heart and blood vessels.

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