Full Paper

Testosterone Relaxes Isolated Human Radial Artery by Potassium Channel Opening Action

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Abstract. Preliminary clinical studies of testosterone therapy in male patients with coronary artery disease obtained promising results. However, little is known about the in vitro effects of testosterone in human isolated arteries. We investigated the effect of testosterone on contractile tone of human isolated radial artery (RA). Testosterone was added (0.1 – 300 μ M) cumulatively to organ baths after precontraction with KCl (45 mM) and phenylephrine (PE, 10 μ M). Testosterone-induced relaxations were tested in the presence of the cyclooxygenase inhibitor indomethacin (10 μ M), nitric oxide synthase inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME, 100 μ M), non-selective large conductance Ca²⁺-activated and voltage-sensitive K⁺ channel inhibitor tetraethylammonium (TEA, 1 mM), ATP-sensitive K⁺ channel inhibitor glibenclamide (GLI, 10 μ M), and voltage-sensitive K⁺ channel inhibitor 4-aminopyridine (4-AP, 1 mM). Testosterone produced relaxation in human RA ($E_{\max}$: $53.03 \pm 2.76\%$ and $66.83 \pm 1.97\%$ of KCl and PE-induced contraction, respectively). Except for GLI, the relaxation to testosterone is affected by neither K⁺ channel inhibitors (TEA, BaCl₂, and 4-AP), L-NAME, nor indomethacin. We report for the first time that supraphysiological concentrations of testosterone induces relaxation in RA. This response may occur in part via ATP-sensitive K⁺ channel opening action.

Keywords: human radial artery, testosterone, vasodilation, potassium channel

Introduction

The risk of developing coronary artery disease and 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 (1, 2). This is reported to result from the unfavourable effects of the male sex hormone, testosterone (3). However, numerous clinical and epidemiological studies reported a controversial relationship between testosterone and cardiovascular disease. For example, low circulating testosterone levels in men are positively correlated with numerous risk factors for coronary heart disease (3, 4). Evidence also supports a beneficial effect of testosterone on the blood lipid profile and against atheroma formation (3, 5–9). Furthermore, acute intracoronary or intravenous infusion of testosterone also provides rapid improvements in

myocardial ischemia (10, 11). Finally, testosterone replacement therapy improves myocardial ischemia in patients with coronary artery disease, an effect presumably due to testosterone-induced coronary vasodilatation (12).

Previous animal studies have demonstrated that testosterone acts as a direct coronary vasodilator in a variety of species, including rabbit, dog, pig, and rat, both in vivo (13) and in vitro (14–20). Testosterone is also reported to exhibit a vasodilatory action in thoracic aorta (14, 21–23) and in vessels isolated from the mesenteric (24) and pulmonary (25, 26) vasculature of experimental animals and more recently in isolated human pulmonary and mesenteric arteries (27). Increasing lines of evidence indicate that the vasodilatory effects of testosterone occur via non-genomic mechanisms. These effects involve primarily the vascular smooth muscle, without requiring the presence of endothelium, although an endothelial contribution is apparent in some arteries from experimental animals such as canine coronary artery (13) and rat mesenteric arterial bed (24). To date, the mechanism behind the vasodilatory action of test-

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osterone is still under debate and might be through either activation of K^+ channels or blockade of Ca^{2+} channels in vascular muscle cells (28).

There is a complex-mixture of vessel-type dependent mechanisms reported in previous studies about the type of the K^+ channel involved in the vasodilatory action of testosterone. In rabbit, dog, and pig coronary conduit arteries, testosterone acts via non-selective large conductance Ca^{2+} -activated potassium channel (BK_{Ca}) and voltage-sensitive potassium channel (K_V) opening action, but testosterone-induced vasodilation occurs via ATP-sensitive potassium channel (K_{ATP}) opening action in dog coronary resistance arteries (13, 14, 20). Accordingly, we have recently shown that testosterone acts via BK_{Ca} opening action in human internal mammary artery (IMA), a conduit vessel used in coronary bypass operations (29).

The radial artery (RA) is the graft of second choice, after IMA, for myocardial revascularization to replace diseased coronary vessels. However, as with any other small vessel, RA has a tendency to spasm during operation and in the early postoperative period. RA shows resistance vessel characteristics as elastomuscular wall structure and a small diameter. There are several studies about the effects of vasoactive substances on RA (30–33), but there is no study on the mechanism of the effect of testosterone-induced vasodilation. We have recently shown that testosterone relaxes IMA and RA, but it has no vasodilatory effect on human umbilical arteries (UA) (29). Therefore, the present study was designed to elucidate the effect of pharmacological concentrations of testosterone (0.1–300 μ M) in human RA and to explore the vascular mechanisms of testosterone response. Our purpose was to test whether or not testosterone-induced vasodilation in RA occurs via K^+ channels similar to resistance arteries.

Materials and Methods

Tissue preparation

Human radial arteries were obtained from patients undergoing bypass surgery. Approval to use discarded RA tissue was granted by the ethics committee of GATA Gulhane Faculty of Medicine and this investigation conforms with the principles outlined in the Declaration of Helsinki. The arteries were placed in oxygenated Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4 , 1.2 mM $MgSO_4$, 10 mM glucose, and 25 mM $NaHCO_3$, pH 7.4) at room temperature and transferred immediately to the laboratory. The arteries were dissected from adhering fat and connective tissue then cut into 3–4-mm-long rings. The rings were mounted in organ baths, each containing

10 ml of Krebs-Henseleit solution, on L-shaped braces for tension measurement along the circumferential axis. The solution was gassed with 95% O_2 and 5% CO_2 at 37°C. Changes in arterial tensions were recorded isometrically by a force-displacement transducer (model FT03, Grass Instruments; Astro-Med, Inc., West Warwick, RI, USA) and recorded continuously on a multichannel recorder polygraph (model P122, Grass Instrument; Astro-Med, Inc.) by using computer software (Polyview, version 2.0, Grass Instruments; Astro-Med, Inc.). The segments were allowed to equilibrate under final resting force of 1.5 g for at least 1.5 h and they were washed every 15 min prior to one of the following four protocols being undertaken.

Experiments with four esterified testosterone analogs

After the equilibration period, arterial rings were challenged with 45 mM KCl to test their viability. The strips that were contracted more than 1.5 g were included in the experimental protocol. The tissues were washed every 10 min during a 30-min additional waiting period. After precontraction with submaximally effective concentrations of depolarizing agent KCl (45 mM) or phenylephrine (PE) (10 μ M) (29, 30), tissues were exposed to 100 μ M of four esterified testosterone analogs (testosterone propionate, testosterone phenylpropionate, testosterone decanoate, testosterone isocaproate). The same volume of vehicle, ethyl alcohol, was also tested.

Difference in relaxation to testosterone between KCl and PE-precontracted rings

In this set of experiments, after the equilibration period, arterial strips were challenged with 45 mM KCl to test their viability. After an additional 30 min with repeated washing every 10 min, the tissues were contracted with KCl (45 mM) or PE (10 μ M), and relaxations to cumulative concentrations of testosterone (0.1–300 μ M) were recorded. Each cumulative concentration was applied after the relaxation to previous concentration reached to a plateau. It normally took 5–6 min of extra period to reach to a plateau at concentrations of 0.1 and 100 μ M. We added our final stock solution (100 μ M) of testosterone three times (300 μ M) to baths and waited for the plateau and it took an extra period of 15 min for the preparations to reach to the plateau. In some experiments, we used high concentration of testosterone (1 mM) and we observed that it did not induce additional relaxation when compared to 300 μ M. Thus, we selected the concentration range of testosterone between 0.1–300 μ M in our experiments. We did not let the final ethyl alcohol concentration in the bath exceed 1.5% (v/v) since it might induce

relaxation itself.

Effect of endothelial denudation on testosterone responses

In this group of experiments, endothelial layers of some RA segments were removed by mechanical rubbing with a needle. After arterial strips were pre-contracted with 45 mM KCl, the relaxation to acetylcholine (ACh, 10 μ M) was used to test the success of endothelial denudation.

Experiments with inhibitors of nitric oxide synthase (NOS), cyclooxygenase (COX) and K⁺ channels

In another set of experiments, after the equilibration period, arterial strips were challenged with 45 mM KCl to test the viability. The strips that were contracted more than 1.5 g were included in the experimental protocol. The tissues were washed every 10 min during a 30-min additional waiting period. Before precontraction with the depolarizing agent KCl (45 mM), the rings were incubated with the COX inhibitor indomethacin (10 μ M), NOS inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME, 100 μ M), non-selective BK_{Ca} and K_V inhibitor tetraethylammonium (TEA, 1 mM), K_{ATP} inhibitor glibenclamide (GLI, 10 μ M), voltage-dependent inward rectifier K⁺ channel (K_{IR}) inhibitor barium chloride (BaCl₂, 30 μ M), or K_V inhibitor 4-aminopyridine (4-AP, 1 mM) for 30 min; and relaxations to testosterone (0.1 – 300 μ M) were recorded.

Experiments on Ca²⁺-channel antagonistic effect of testosterone

To investigate the Ca²⁺-channel antagonistic effect of testosterone, concentration-response curves to CaCl₂ (10 μ M – 30 mM) were obtained in the absence and in the presence of testosterone (100 μ M) and the L-type Ca²⁺-channel blocker nifedipine (1 μ M). After RA rings were allowed to equilibrate for 30 min, the rings were washed three times at 10-min intervals with Ca²⁺-free Krebs solution containing 1 mM ethylenediaminetetraacetic acid (EDTA, disodium salt). The rings were then bathed with Ca²⁺-free (containing 1 mM EDTA), high KCl (45 mM) Krebs solution with or without testosterone (100 μ M) for 30 min. In vehicle-control experiments, absolute ethanol was added in the same volume as testosterone. For comparison, nifedipine (1 μ M), instead of testosterone, was assayed in adjacent segments in parallel.

Chemicals

KCl and TEA were purchased from Merck Co (Darmstadt, Germany). Testosterone propionate, testosterone phenylpropionate, testosterone decanoate, and

testosterone isocaproate were gifts from Organon (Ankara, Turkey). PE, EDTA, BaCl₂, GLI, indomethacin, and L-NAME were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 4-AP was purchased from Acros Organics (Morris Plains, NJ, USA). Nifedipine was purchased from Fako Drug Co. (Istanbul, Turkey).

Statistical evaluations

Testosterone-evoked responses are expressed as the arithmetic mean $\pm$ S.E.M. of the % of the KCl or PE response in each corresponding tissue. Statistical significance between two groups was evaluated by Student's *t*-test for paired data. Comparison among multiple groups were made by using a one way ANOVA, followed by Scheffe test post hoc to determine significant differences among the means of the data groups. A probability of *P* < 0.05 was accepted as significant difference.

Results

Four esterified analogs of testosterone (100 μ M) produced relaxation in RA (12.1% – 58.8% of KCl-induced contraction). At this single high concentration, testosterone propionate showed the highest efficacy (42.41 $\pm$ 3.58% of KCl-induced contraction, *n* = 7) among the other testosterone analogs in RA. After precontraction with KCl, the mean value of maximal relaxations with testosterone phenylpropionate, testosterone decanoate, and testosterone isocaproate were 30%, 27%, and 17%, respectively. Thus, we used testosterone propionate in all experiments.

Cumulative concentrations of testosterone (0.1 – 300 μ M) elicited concentration-dependent relaxation of 45 mM KCl-induced active tone in RA (*E*_{max}: 53.03 $\pm$ 2.76% of KCl contraction, *n* = 17). In PE-precontracted RA rings, the sensitivity to testosterone seemed to be similar to KCl-precontracted rings (Fig. 1), whereas the maximum testosterone relaxation of PE-induced active tone was significantly higher than that of KCl-induced precontraction (*E*_{max}: 66.83 $\pm$ 1.97% of PE contraction, *P* < 0.01, *n* = 5). In addition, testosterone-induced relaxation in PE-precontracted RA rings was significantly higher than that of KCl-precontracted rings at all concentrations tested except for the lowest one (0.1 μ M). The baseline values for the level of precontraction (1.43 $\pm$ 0.07 and 1.39 $\pm$ 0.06 g in KCl and PE experiments, respectively) and the active tone induced by precontractile agents 6.29 $\pm$ 0.37 and 6.64 $\pm$ 0.82 g in KCl and PE experiments, respectively) were similar. The highest concentration of vehicle (ethanol, 1.5%) had no significant relaxant effect (3%, *n* = 4; Fig. 2). Time-control experiments showed no spontaneous relaxation

Fig. 1. Cumulative relaxation to testosterone (0.1–300 μ M) in human radial artery rings precontracted with 45 mM KCl (open square, $n = 17$) and 10 μ M phenylephrine (solid square, $n = 5$). Data are expressed as % relaxation of tone. Each value is a mean $\pm$ S.E.M., and vertical lines represent S.E.M. * $P < 0.05$ and ** $P < 0.01$ vs KCl Control, unpaired Student's t -test.

A. Time Control

B. Testosterone (μ M)

C. Vehicle (Ethanol, 30 μ l)

Fig. 2. Original tracings of KCl (45 mM)-precontracted human radial artery rings. A: time-control experiment in a precontracted artery without adding testosterone. B: relaxation to testosterone. C: vehicle has no significant relaxant effect.

in precontracted arteries without adding testosterone ($n = 4$, Fig. 2).

Mechanical rubbing of the endothelium did not significantly modify relaxation induced by testosterone (0.1–300 μ M). In contrast, relaxation induced by ACh (10 μ M) was significantly reduced in endothelium-free

Fig. 3. Effects of the nitric oxide synthase inhibitor L-NAME (100 μ M, $n = 6$) and the cyclo-oxygenase inhibitor indomethacin (10 μ M, $n = 5$) on the cumulative relaxation to testosterone (0.1–300 μ M) in human radial artery rings precontracted with 45 mM KCl. Data are expressed as % of the response to 45 mM KCl-induced contraction. Vertical lines represent S.E.M.

RA (data were not shown). Incubation of RA with the NOS inhibitor L-NAME (100 μ M) and COX inhibitor indomethacin (10 μ M) did not significantly modify relaxation to testosterone (Fig. 3).

Relaxation to testosterone (0.1–300 μ M) was not significantly modified by incubation of RA with the non-selective BK_{Ca} and K_V inhibitor TEA (1 mM), K_{IR} inhibitor barium chloride (30 μ M), or K_V inhibitor 4-AP (1 mM) (Fig. 4: A–C). On the other hand, the K_{ATP} inhibitor GLI (10 μ M) was able to significantly modify testosterone-induced relaxation significantly ($P < 0.001$ at 300 μ M) (Fig. 4D).

$CaCl_2$ (10 μ M–30 mM) caused contraction in a concentration-dependent-manner in RA rings depolarized by 45 mM KCl. Pre-incubation with 1 μ M nifedipine significantly ($P < 0.05$, $P < 0.01$) inhibited $CaCl_2$ -induced contraction. However, testosterone inhibited contractions induced by $CaCl_2$ slightly but not significantly (Fig. 5).

Fig. 5. Effects of testosterone (100 μ M) and nifedipine (1 μ M) on CaCl₂-induced contraction in RA depolarized by 45 mM KCl. Data are expressed as % of the response to 45 mM KCl-induced contraction. Vertical lines represent S.E.M. **P* < 0.05 and ***P* < 0.01 vs vehicle control, ANOVA.

Discussion

The present study is the first to report male sex hormone testosterone-induced relaxation of RA in vitro. After application of a single high concentration (100 μ M), testosterone propionate showed the highest efficacy among the esterified testosterone analogues. Testosterone propionate (0.1–300 μ M) caused potent and rapid vasorelaxation in RA. The maximum magnitude of testosterone-induced relaxation in RA (50%–60%) is smaller than those (80%–100%) observed in animal vessels (27). The acute vasorelaxant effect of testosterone is not mediated via endothelium-dependent NO and prostanoid-mediated mechanisms. However, vasorelaxation to testosterone seems to be mediated in part by increasing potassium efflux through K_{ATP}

Fig. 4. Cumulative relaxation to testosterone (0.1–300 μ M) in human radial artery rings precontracted with 45 mM KCl in the presence of voltage-sensitive K⁺ channel inhibitor 4-aminopyridine (1 mM, n = 7) (A); large conductance Ca²⁺-activated K⁺ channel and voltage-sensitive K⁺ channel inhibitor tetraethylammonium (1 mM, n = 5) (B); voltage-dependent inward rectifier K⁺ channel inhibitor barium chloride (BaCl₂, 30 μ M, n = 6) (C); and ATP-dependent K⁺ channel blocker glibenclamide (10 μ M, n = 8) (D). Data are expressed as % of the response to 45 mM KCl-induced contraction. Vertical lines represent S.E.M. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs testosterone control, paired Student's *t*-test.

channels, but not via BK_{Ca}, K_{IR}, or K_V channels.

Previously, it was reported that the relative polarity of the testosterone molecule determines its vasorelaxation efficacy (23). Testosterone propionate is the most polar, least lipid-soluble, and smallest molecular weight (MW: 344.5) among the esterified testosterone analogs tested in the present study. Thus, our data support the suggestion that testosterone-induced vasorelaxation is a non-genomic and structurally specific effect of the testosterone molecule, which is enhanced in more polar analogues that have a lower permeability to the vascular smooth muscle cell membrane (23).

The results of the present study clearly demonstrate that testosterone-induced vasodilation in RA is endothelium-independent and it does not involve vasodilator prostaglandins and NO. In our study, identical concentration-response curves were obtained following exposure to both the COX inhibitor indomethacin and NOS inhibitor L-NAME. In addition, relaxation to testosterone was not different in endothelial denuded RA segments. Previously, a number of studies have investigated the involvement of dilator prostanoids and endothelium-derived NO (13, 14, 22–24, 26, 34, 35). However, some of these studies report that indomethacin, an inhibitor of COX responsible for the synthesis of the cyclic endoperoxide from arachidonate, has no inhibitory effect on testosterone-mediated relaxation (13, 14, 22, 26). Similarly, testosterone-induced vasodilation has been reported to be preserved in endothelial denuded vessels (14–17, 20–22, 36) or in the presence of inhibitors of NOS or guanylate cyclase (14, 20, 22, 26). In addition, previous studies also showed that testosterone had no effect on NOS activity in human and bovine aortic endothelial cells (36, 37).

In our study, in PE-precontracted RA rings, testosterone-induced relaxation was significantly higher than that of KCl-precontracted rings. Previously, the study of Deenadayalu et al. (20) reported a decrease in the response to testosterone under similar conditions with our study, namely less testosterone relaxation after KCl-precontraction compared to that after prostaglandin F_{2α}-precontraction. An explanation of this situation may lie with the potassium channel function which is compromised in conjunction with the vasodilatory efficacy of testosterone. In the present study, significant reduction in the efficacy of testosterone in KCl-precontracted arteries demonstrate that testosterone may be acting to open potassium channels as has been previously suggested (20, 24).

We showed that testosterone-induced vasorelaxation was reduced by GLI (a K_{ATP} inhibitor), but not by TEA (a BK_{Ca} and K_V inhibitor), BaCl₂ (a K_{IR} inhibitor), or 4-AP (a K_V inhibitor). These results suggest that testos-

terone may cause vasorelaxation via activation of K_{ATP} channels in RA. In agreement with our observations, Deenadayalu et al. (20) recently demonstrated that TEA inhibited vasorelaxation to testosterone in pig coronary arteries. In addition, Deenadayalu et al. (20) confirmed that testosterone opened BK_{Ca} channels in single pig coronary myocytes by direct evidence from patch-clamp studies. In contrast, Chou et al. (13) and Honda et al. (22) previously showed that the K_{ATP}-channel inhibitor GLI reduced vasorelaxation to testosterone in dog coronary artery and rat thoracic aorta, respectively. However, Ding and Stallone (23) demonstrated that neither incubation with GLI nor TEA had any effect on the vasodilatory response to testosterone in rat thoracic aorta. However, they showed that incubation with 4-AP significantly reduced vasorelaxation to testosterone and they therefore proposed that testosterone acts via K_V channel opening action. In our study, reduction in the efficacy of testosterone in vessels precontracted with KCl and the inhibitory effect of GLI on vasorelaxation to testosterone together demonstrate that testosterone-mediated vasodilation may occur via activation of K_{ATP} channels. The remaining relaxation to testosterone, after GLI inhibition, may be due to a direct inhibitory effect of testosterone on Ca²⁺ influx because we observed that testosterone (100 μM) slightly inhibited CaCl₂-induced contractions in RA. Consistent with our observation, it has been recently demonstrated that testosterone at high concentration inhibits Ca²⁺ influx in rat aorta (35).

Previous studies have suggested that testosterone acts via BK_{Ca} and K_V channel opening action in rabbit, dog, and pig coronary conduit arteries (13, 14, 20). Accordingly, we have recently shown that testosterone acts via BK_{Ca} channel opening action in an human conduit artery, the IMA (29). In the present study, however, testosterone-induced vasodilation in RA seems to occur via K_{ATP} channel opening action, similar to dog coronary resistance arteries (13). RA shows resistance vessel characteristics as elastomuscular wall structure and a small diameter. Therefore, it may be suggested that testosterone preferentially stimulates the K_V and K_{Ca} channels in large conductance vessels and stimulates the K_{ATP} channel in small resistance vessels. In fact, there are some differences in the vascular reactivity between large conductance and small resistance arteries. For example, it was reported that adenosine, a metabolic regulator of coronary blood flow (probably via activating K_{ATP} channels and inhibiting L-type Ca²⁺ current), preferentially blocked the action potential in the small coronary artery, but had no effect on the action potential in the large coronary artery (38). Similarly, the coronary dilating actions of a K_{ATP}-channel-opening drug, levromakalim, were heterogeneous, and small resistant coro-

nary arteries were more sensitive to this drug (39). It was also reported that the resting tone of large conductance vessels was little affected by GLI, whereas that of small resistance vessels was affected (40, 41). Therefore, the tone of the small vessels is likely to be regulated by the glibenclamide-sensitive K_{ATP} channel (42). Another important finding is that testosterone relaxes IMA and RA but it has no vasodilatory effect on UA (29, 43). Human UA is unique since it shows conductance vessel characteristics like elastic wall structure and it has a small diameter. Functional K_V and BK_{Ca} channels are present in UA (44). However, this artery seems to lack the non-genomic signaling pathway involved in testosterone-induced vasodilation.

An important point of the present and the previous studies is the latency for the vasodilating effect of testosterone. In most of the mechanistic studies, free testosterone analogues were used, and the vasodilatory action of testosterone was rapid, occurring within minutes. Accordingly, we have observed the vasodilatory effect of testosterone within minutes in the present study. Previously, it was shown that the covalent linkage of testosterone analogues to a macromolecule, such as albumin, had no effect on the timescale and magnitude of the vasodilatory response to testosterone (23, 45). Therefore, the conjugation of testosterone to albumin eliminates the permeability of testosterone to the cell membrane, and thereby, these findings again suggest a non-genomic action.

The issue of how testosterone is solubilized and delivered is of great importance. Different studies in humans and animals have used different solvents in different concentrations (e.g. ethanol and dimethylsulphoxide). Solvents have unselective effects on ion channel activity (46), within milliseconds, which could interfere with the selective actions of testosterone. In previous studies however, no significant relaxant effect of final concentrations of the solvents has been reported (13, 14, 22–26, 34, 35). In our experiments, we did not let the final ethanol concentration in the bath exceed 1.5% (v/v) since it might induce relaxation itself (29).

Taken together, our findings provide evidence for the marked vasodilating effect induced by testosterone in RA. The discrepancy among the previous studies in proposing a vasodilatory mechanism for testosterone may be due to the variance in the species and the vessels studied since we also observed testosterone-induced relaxation in IMA but not UA (29, 43). Previously, Fausett et al. also reported that testosterone had no relaxant effect on UA (47). Further studies, especially in different human vascular preparations and with cellular patch-clamp techniques, are essential to understand the exact mechanism of the effect of testosterone and to

fully realize the therapeutic potential of testosterone therapy in male patients with coronary artery disease.

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