Testosterone-induced relaxation of rat aorta is androgen structure specific and involves K⁺ channel activation

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Ding, Andrew Q., and John N. Stallone. Testosterone-induced relaxation of rat aorta is androgen structure specific and involves K⁺ channel activation. J Appl Physiol 91: 2742–2750, 2001.—Recent studies have established that testosterone (Tes) produces acute (nongenomic) vasorelaxation. This study examined the structural specificity of Tes-induced vasorelaxation and the role of vascular smooth muscle (VSM) K⁺ channels in rat thoracic aorta. Aortic rings from male Sprague-Dawley rats with (Endo+) and without endothelium (Endo−) were prepared for isometric tension recording. In Endo− aortas precontracted with phenylephrine, 5–300 μM Tes produced dose-dependent relaxation from 10 μM (4 ± 1%) to 300 μM (100 ± 1%). In paired Endo+ and Endo− aortas, Tes-induced vasorelaxation was slightly but significantly greater in Endo+ aortas (at 5–150 μM Tes); sensitivity (EC₅₀) of the aorta to Tes was reduced by nearly one-half in Endo− vessels. Based on the sensitivity (EC₅₀) of Endo− aortas, Tes, the active metabolite 5α-dihydrotestosterone, the major excretory metabolites androsterone and etiocholanolone, the nonpolar esters Tes-enanthate and Tes-hemisuccinate (THS), and THS conjugates to BSA (THS-BSA) exhibited relative potencies for vasorelaxation dramatically different from androgen receptor-mediated effects observed in reproductive tissues, with a rank order of THS-BSA > Tes > androsterone = THS = etiocholanolone > dihydrotestosterone ≈ Tes-enanthate. Pretreatment of aortas with 5 mM 4-aminopyridine attenuated Tes-induced vasorelaxation by an average of 44 ± 2% (25–300 μM Tes). In contrast, pretreatment of aortas with other K⁺ channel inhibitors had no effect. These data reveal that Tes-induced vasorelaxation is a structurally specific effect of the androgen molecule, which is enhanced in more polar analogs that have a lower permeability to the VSM cell membrane, and that the effect of Tes involves activation of K⁺ efflux through K⁺ channels in VSM, perhaps via the voltage-dependent (delayed-rectifier) K⁺ channel.

tenothelium; endothelium-dependent vasodilation; endothelium-independent vasodilation; vasorelaxation; vasodilation

Numerous clinical and epidemiological studies have established that marked sexual dimorphism exists in a variety of human cardiovascular diseases. For example, coronary artery disease and hypertension occur more frequently in men than in premenopausal women (24, 38), whereas Reynaud’s disease (5) and primary pulmonary hypertension (39) occur more frequently in premenopausal women than in men. Furthermore, hypertensive men and premenopausal women are both reported to have lower plasma androgen and higher plasma estrogen concentrations, respectively, than their normotensive counterparts (17, 21). These sex differences in cardiovascular disease and in plasma gonadal steroid hormone concentrations suggest that these hormones influence vascular function and the development of hypertension.

Several recent studies have documented direct vasodilatory effects of estrogen on the vasculature involving rapid, presumably nongenomic mechanisms of action. These rapid vasodilatory effects of estrogen occur within minutes and have been attributed to endothelial (6, 12, 43) and/or vascular smooth muscle (VSM) mechanisms (6, 12, 19, 27, 35). Previous studies have provided a varied evidence of the existence of rapid, nongenomic mechanisms of action of estrogen in cardiac, neural, and vascular tissues (15, 18, 26, 34, 41).

In comparison, little information is available on the direct effects of testosterone (Tes) on the vasculature. Yue et al. (44) demonstrated an acute vasodilatory effect of Tes in the rabbit aorta and coronary artery in vitro that is endothelium (Endo) independent and may involve activation of VSM K⁺ channels. In contrast, recent studies of the rat aorta established that Tes produces acute vasorelaxation that is gender and androgen receptor independent and involves both Endo-dependent (nitric oxide) and -independent mechanisms of action (7). Similar Endo-dependent (nitric oxide) and -independent vasodilatory effects of Tes in canine coronary conductance and resistance arteries in vivo were reported by Chou et al. (4). A variety of recent studies on the rapid, nongenomic effects of both estrogen and Tes suggest that the acute, Endo-independent vasodilatory effects of Tes on VSM may involve activation of K⁺ channels in VSM.
Thus the purpose of the present study was to determine the structural specificity of the Endo-dependent and -independent vasodilatory effects of Tes in the rat aorta by examining the ability of selected androgen analogs and metabolites to produce vasorelaxation. To determine the role of VSM K\(^+\) channels in this acute, nongenomic effect of Tes, the effect of VSM K\(^+\) channel inhibition on the Endo-independent vasodilatory effect of Tes was also examined.

**METHODS**

**Experimental Animals**

Male Sprague-Dawley rats (12–16 wk of age) were obtained from Zivic-Miller Laboratories (Zelienople, PA). All rats were housed in the Northeastern Ohio University College of Medicine Comparative Medicine Unit vivarium facilities. Temperature (21–26°C) and lighting (12:12-h light-dark cycle) were controlled. Purina laboratory chow (Purina Mills, St. Louis, MO) and tap water were provided ad libitum. All experimental procedures used in these studies were reviewed and approved by the Northeastern Ohio University College of Medicine Institutional Animal Care and Use Committee.

**Preparation of Vascular Tissue**

Thoracic aortas were removed from rats after death by decapitation and placed in chilled (4°C) Krebs-Henseleit-bicarbonate (KHB) solution gassed with 95% O\(_2\)-5% CO\(_2\). Composition of the KHB solution was (in mM) 118.0 NaCl, 25.0 NaHCO\(_3\), 10.0 glucose, 4.74 KCl, 2.50 CaCl\(_2\), 1.18 MgSO\(_4\), and 1.18 KH\(_2\)PO\(_4\) (pH 7.40, osmolality = 292 ± 1 mosmol/kgH\(_2\)O). Aortas were cleaned of all adipose and connective tissue, and the midthoracic region was cut into rings (3 mm long). During preparation of the rings, care was taken to avoid stretching the tissue or touching the luminal surface to preserve endothelial integrity, which was evaluated functionally in all experiments (as described below). To assess the role of the Endo in the vascular responses to Tes and its analogs, some aortas were denuded before mounting by gently rubbing the luminal surface with a frayed nylon string (36). After preparation, the aortic rings were mounted on two 25-gauge stainless steel wires; the lower one was attached to a stationary stainless steel rod, and the upper one was attached to a force-tension transducer (FT-03D, Grass Instruments, Quincy, MA) connected to a polygraph (2600S, Gould, Cleveland, OH) for continuous recording of aortic tension. Immediately after mounting, the aortas were suspended in water-jacketed tissue baths containing 15.0 ml of KHB warmed (37°C) and continually gassed with 95% O\(_2\)-5% CO\(_2\). Passive tension was gradually adjusted (over 30 min) to 2.50 g (optimal tension for male rat aortas; Ref. 37), and the aortas were equilibrated for 90 min. During this time, the bath solution in the tissue baths was replaced with freshly gassed, warmed KHB every 20 min. After equilibration, the aortas were stabilized with a near-maximal contraction to phenylephrine (PE; 1.0 \(\mu\)M). After the rings achieved a stable contractile tension, the Endo-dependent vasodilator ACh (0.1 \(\mu\)M) was added to the baths to assess endothelial integrity. The tissue baths were then rinsed twice with KHB, and the aortas were allowed to reequilibrate (30 min) before further experimentation.

**Effects of Tes Analogs and Metabolites on Vasorelaxation**

To determine the role of K\(^+\) channel activation in Tes-induced vasorelaxation, antagonists tetraethylammonium (TEA), glybenclamide (Gly), and 4-aminopyridine (4-AP) were employed to determine the contributions of Ca\(^{2+}\)-dependent (K\(_{Ca}\)), ATP-dependent (K\(_{ATP}\)), or voltage-dependent (delayed rectifier, K\(_V\); Ref. 1) K\(^+\) channels, respectively. Although these compounds are not entirely specific for these \(K^+\) channels, at the concentrations used in these studies, these antagonists are relatively selective for the K\(_{Ca}\), K\(_{ATP}\), and K\(_V\) channels, respectively, and are widely used in studies of vascular K\(^+\) channel function (29). The effect of 80 mM KCl on Tes-induced relaxation of the Endo-denuded aorta was also examined.

**Effect of TEA on Tes-induced vasorelaxation.** After the initial stabilization and reequilibration periods, paired Endo–aortas were precontracted with PE (1.0 \(\mu\)M). After a stable contractile tension was attained, TEA (final bath concentration, 1 mM) or its vehicle control (KHB) was added to the baths containing the paired aortas. After a 30-min pre-treatment period, Tes was added to the baths in a cumulative manner to obtain a concentration response for each ring (5–300 \(\mu\)M; because of the expense of this compound). Vascular relaxation responses to Tes and its analogs were calculated as for the Tes analog experiments above.

**Effect of Gly on Tes-induced relaxation.** Paired Endo–aortas were prepared and precontracted with PE (1.0 \(\mu\)M) as for the TEA experiments above. After a stable contractile tension was attained, the paired aortas were pretreated with Gly (10 \(\mu\)M) or its vehicle control (KHB) for 30 min, and a cumulative concentration response to Tes was obtained. Vascular relaxation responses were calculated as for the TEA experiments above.

**Effect of 4-AP on Tes-induced relaxation.** Paired Endo–aortas were prepared and precontracted with PE (1.0 \(\mu\)M) as for the TEA experiments above. After a stable contractile tension was attained, the paired aortas were pretreated with 4-AP (5 mM) or its vehicle-control (KHB) for 30 min, and a cumulative concentration response to Tes was obtained. Vascular relaxation responses were calculated as for the TEA experiments above.

**Effect of 80 mM KCl on Tes-induced vasorelaxation.** After the initial stabilization and reequilibration periods, Endo–aortas were precontracted with PE (1.0 \(\mu\)M), PGP\(_{2\alpha}\) (1.0 \(\mu\)M), or 80 mM KCl to similar active tension (~4,300 mg). After a
stable contractile tension was attained, Tes was added to the baths at a concentration near the EC50 for Tes in PE-precontracted aortas. Vascular relaxation responses to Tes were calculated as a percentage of the PE-, PGF2α-, or KCl-induced precontraction.

**Genomic Effects of THS vs. THS-BSA Conjugate in Seminal Vesicles**

To compare the genomic effects of THS vs. THS-BSA conjugate in an established androgen target tissue (32), the effects of these androgen analogs on DNA synthesis in the rat seminal vesicle were determined. Genomic DNA was isolated from rat seminal vesicles using the Trizol reagent (GIBCO-BRL, Grand Island, NY), as described in the Trizol protocol, with minor modifications, as described below. Seminal vesicles were removed from rats (under sterile conditions) after death by decapitation. To maximize responsiveness of this target tissue to the androgen analogs, the rats underwent bilateral orchietomy (under pentothal sodium anesthesia, 60 mg/kg ip) 4 days before removal of the tissue. The seminal vesicles were finely cross sectioned (2–3 mm), and the tissue was then incubated in 5 ml of MEM alone (control) or in the presence of THS (3 or 30 ng/ml), THS-BSA conjugate (3 or 30 ng/ml), or BSA (3 ng/ml) for 48 h at 37°C in a humidified CO2 incubator (5% CO2; Lab-Line Instruments, Melrose Park, IL). The incubation mixtures were changed at 24 h and after 48 h; the tissue was removed from the media, weighed, and homogenized in 1–2 ml of Trizol reagent using a Polytron (Brinkmann Instruments, Westbury, NY). The samples were centrifuged briefly (10 min at 12,000 g at 4°C) to remove insoluble material from the homogenate. The supernatants were then transferred to clean, sterile microcentrifuge tubes. Chloroform (200 μl/ml of Trizol) was added to each tube, and the samples were then shaken, incubated at room temperature for 5 min, and then centrifuged (15 min at 12,000 g at 4°C). After the aqueous layer was removed, absolute ethanol (EtOH, 300 μl/ml of Trizol) was added to the samples to precipitate the DNA. The tubes were gently inverted several times, incubated at room temperature for 3 min, and then centrifuged (2,000 g for 5 min at 4°C). The supernatant was carefully removed, and the pellet was washed three times with 0.1 M sodium citrate in 10% EtOH (1.5 ml/ml Trizol) and stored at room temperature for 15 min before recentrifugation (2,000 g for 5 min at 4°C). The supernatant was removed, and the pellets were allowed to air dry for 10 min before dissolution in 8 mM NaOH and centrifugation (12,000 g for 10 min at 4°C) to remove insoluble material. The supernatant was then transferred to clean, sterile tubes, and the DNA concentration was determined by measuring the absorbance at 260 nm. Tissue DNA content was normalized by tissue weight and is expressed as nanograms DNA per milligrams tissue.

**Chemical Reagents and Drug Preparation**

4-AP, ACh chloride, apamin, Gly, and PE hydrochloride were purchased from Sigma Chemical (St. Louis, MO). PGF2α was obtained from Cayman Chemical (Detroit, MI). And (5α-androstane-3α-ol-17-one), DHT (5α-androstane-17β-ol-3-one), Etio (5β-androstane-3α-ol-17-one), TEN (4-androsten-17β-ol-3-one enanthate), Tes (4-androsten-17β-ol-3-one), THS (4-androsten-17β-ol-3-one hemisuccinate), and THS-BSA conjugate (4-androsten-17β-ol-3-one hemisuccinate BSA) were obtained from Steraloids (Wilton, NH). TEA chloride was obtained from Aldrich Chemical (Milwaukee, WI). All drug solutions were prepared fresh daily (4-AP, ACh, apamin, PE, Gly, TEA, THS-BSA) or diluted from stock solutions stored at −20°C (PGF2α, And, DHT, Etio, TEN, Tes, THS); these working solutions were kept on ice during the experiments. Stock solutions were prepared in KHB (4-AP, apamin, TEA, Gly), KHB with 100 μM ascorbic acid (PE), distilled water (ACh), 20 mM Na2CO3 (PGF2α), 50% EtOH (And, DHT, Etio, TEN, Tes, THS), or 5% DMSO-2% Solutol in distilled water (THS-BSA). Stock solutions of 4-AP were adjusted to physiological pH before dilution in tissue bath KHB.

**Data Analysis**

All data are expressed as means ± SE; n indicates the number of animals studied. Vasorelaxation responses to Tes or its analogs were calculated as percentages of the PE precontraction. The concentration of Tes and its analogs producing EC50 was calculated individually from the log concentration-response curve of each aortic ring and reported as the geometric mean ± SE for each experimental group. In the androgen analog experiments, the same concentration-response data for Tes were plotted with the concentration-response data for each of the analogs, to allow direct comparison. Paired concentration-response data (K+ channel and Endo+/Endo− Tes analog experiments) were compared by paired t-test at each concentration of Tes or analog. Multiple group concentration-response data were compared by one-way analysis of variance among experimental groups (e.g., genomic effects of THS vs. THS-BSA) or by two-way analysis of variance (e.g., effect of Endo vs. Tes analog) to detect significant differences, followed by paired or unpaired t-tests to distinguish significant differences among the means of the experimental groups. To correct for the increase in type I error associated with multiple comparisons, a Bonferroni modification of the t-test was employed (20).

**RESULTS**

**Effects of Tes Analogs and Metabolites on Vasorelaxation**

Tes-induced vasorelaxation was slightly but significantly greater in Endo+ than in Endo− aortas (P < 0.001); this effect of the Endo was significant at 5–150 μM Tes (Fig. 1A). The sensitivity (EC50) of the rat aorta to Tes was reduced by nearly 50% in Endo− aortas at the lowest (5–150 μM) concentration (P < 0.0001; Fig. 1A, Table 1). Similarly, the major nonpolar excretory metabolite Etio also produced significantly greater relaxation in Endo+ aortas at all concentrations studied (P ≤ 0.024). Both the sensitivity and maximal response of the aorta to Endo+ were greater in Endo+ than in Endo− aortas (P = 0.016; Fig. 1A, Table 1). The other major nonpolar excretory metabolite Etio produced significantly greater (P < 0.017) vasorelaxation in Endo+ than in Endo− aortas at the lowest concentrations (5–10 μM); however, neither sensitivity nor maximal response differed significantly between Endo+ and Endo− aortas (P > 0.05; Fig. 1B, Table 1). The major biologically active metabolite DHT produced slightly but significantly (P = 0.044) greater vasorelaxation in Endo+ aortas at the lowest (5–10 μM) and highest (150–300 μM) concentrations; although maximal response was greater in the presence of the Endo (P ≤ 0.001), sensitivity was somewhat variable and did not vary between Endo+ and Endo− aortas (P > 0.05; Fig. 2A, Table 1).

The Tes ester analogs TEN and THS produced significantly less vasorelaxation of the rat aorta than did...
Tes and exhibited less Endo-dependent effects. Compared with Tes, the aorta exhibited a significantly lower sensitivity and maximal response to THS (P ≤ 0.006), and THS-induced vasorelaxation did not differ significantly between Endo+ and Endo− aortas throughout the concentration range (P > 0.05; Fig. 2B, Table 1). The aorta exhibited a dramatically lower sensitivity and maximal vasorelaxation response to TEN compared with Tes. Although TEN produced slightly but significantly (P ≤ 0.019) greater vasorelaxation in Endo+ than in Endo− aortas at lower concentrations (5–25 μM), neither sensitivity nor maximal response differed significantly between Endo+ and Endo− aortas (P > 0.05; Fig. 2B, Table 1).

In marked contrast, conjugation of THS to BSA (THS-BSA) dramatically enhanced the vasorelaxation activity of this analog, enhancing both the sensitivity and maximal response of Endo+ and Endo− aortas compared with THS alone (P ≤ 0.014; Figs. 2B and 3, Table 1). THS-BSA-induced vasorelaxation did not differ significantly (P > 0.05) between Endo+ and Endo− aortas. Interestingly, both Endo+ and Endo− aortas exhibited a sensitivity to THS-BSA-induced vasorelaxation that was comparable to the sensitivity of Endo+ aortas to Tes (Table 1). In Endo− aortas, THS-BSA produced significantly more vasorelaxation than Tes (P ≤ 0.038) at lower concentrations (10–50 μM) but similar effects (P > 0.05) at higher concentrations (75–150 μM; Fig. 3). In comparison, Endo− aortas exhibited a substantially lower sensitivity and maximal response to THS alone than to either THS-BSA or Tes (Fig. 3, Table 1).

Relative Potencies of Tes Analogs and Metabolites to Produce Vasorelaxation

Tes and its analogs and metabolites exhibit substantial differences in their ability to produce vasorelaxation of the Endo-denuded rat aorta (Figs. 1–3, Table 1). Based on the sensitivity (EC50) of the Endo− aorta to androgen-induced vasorelaxation, the rank order potencies of the various Tes analogs is as follows: THS-BSA > Tes > And = THS = Etio > DHT >> TEN. A slightly different rank order exists, based on the maximal relaxation responses of the Endo− aorta: Tes > Etio > THS-BSA = And > THS > DHT >> TEN (Table 1). Despite these marked differences in the sensitivity of the aorta to Tes analogs and metabolites, the time courses of the vasorelaxation responses were quite similar among Tes and all analogs and metabolites studied.

Effects of TEA, 4-AP, Gly, and 80 mM KCl on Tes-induced Vasorelaxation

There were no significant differences in Tes-induced vasorelaxation between vehicle control and either

<table>
<thead>
<tr>
<th>Androgen Analog</th>
<th>n</th>
<th>Treatment</th>
<th>Maximal Relaxation, %</th>
<th>EC50, μM</th>
</tr>
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<tbody>
<tr>
<td>Testosterone</td>
<td>8</td>
<td>Endo+</td>
<td>100 ± 0.0a</td>
<td>40.2 ± 2.0fg</td>
</tr>
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<td></td>
<td>8</td>
<td>Endo−</td>
<td>100 ± 0.0a</td>
<td>59.5 ± 3.1b</td>
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<tr>
<td>5α-Dihydrotestosterone</td>
<td>8</td>
<td>Endo+</td>
<td>68.9 ± 3.5e</td>
<td>188 ± 34.7</td>
</tr>
<tr>
<td>(active metabolite)</td>
<td>8</td>
<td>Endo−</td>
<td>57.2 ± 3.0e</td>
<td>225 ± 21.8</td>
</tr>
<tr>
<td>Androsterone (excretory metabolite)</td>
<td>8</td>
<td>Endo+</td>
<td>91.6 ± 1.6b</td>
<td>84.1 ± 5.7f</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Endo−</td>
<td>83.9 ± 2.9h</td>
<td>119 ± 5.9k</td>
</tr>
<tr>
<td>Etiocholanolone (excretory metabolite)</td>
<td>8</td>
<td>Endo+</td>
<td>94.5 ± 1.1d</td>
<td>135 ± 6.5k</td>
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<tr>
<td></td>
<td>8</td>
<td>Endo−</td>
<td>94.3 ± 1.0d</td>
<td>131 ± 7.1k</td>
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<tr>
<td>Testosterone enanthate (ester analog)</td>
<td>6</td>
<td>Endo+</td>
<td>25.0 ± 2.3k</td>
<td>6.483e</td>
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<td>Testosterone hemisuccinate (ester analog)</td>
<td>6</td>
<td>Endo−</td>
<td>22.6 ± 1.7j</td>
<td>7.494f</td>
</tr>
<tr>
<td>Testosterone hemisuccinate-BSA (BSA, conjugate)</td>
<td>6</td>
<td>Endo+</td>
<td>98.6 ± 3.7j</td>
<td>34.7 ± 6.5g</td>
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<tr>
<td></td>
<td>6</td>
<td>Endo−</td>
<td>98.5 ± 4.5j</td>
<td>43.7 ± 6.0</td>
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</table>

Values are means ± SE; n = no. of animals. Endo+, endothelium intact; Endo−, endothelium denuded. *EC50 values estimated by extrapolation. Mean values without common superscript are significantly different for *EC50-maximal relaxation (0.0155 ≤ P ≤ 0.0001) and *=EC50 (0.0132 ≥ P ≥ 0.0001). All analogs were studied at 0–300 μM, except testosterone hemisuccinate-BSA (0–150 μM).
TEA- or Gly-treated aortas (Fig. 4, B and C). The absence of Tes effects on K<sub>Ca</sub> channels was further confirmed in additional experiments using a more selective inhibitor of the small-conductance K<sub>Ca</sub> channel apamin (0.5 μM), which also failed to alter Tes-induced vasorelaxation (n = 3; data not shown). In contrast, pretreatment of aortas with 4-AP significantly reduced Tes-induced vasorelaxation at all concentrations of Tes (P < 0.01; Fig. 4A) and reduced the sensitivity to Tes by twofold (100.8 ± 4.2 vs. 51.6 ± 5.3 μM; P < 0.0001). The reductions in Tes-induced relaxation by 4-AP averaged 44.1 ± 1.9% over the concentration range of 25–300 μM. Relaxation responses to 50 μM Tes were reduced substantially (P < 0.008) in aortas precontracted with 80 mM KCl, compared with those precontracted with either PGF<sub>2α</sub> or PE (Table 2).

Genomic Effects of THS vs. THS-BSA in Seminal Vesicles

The effects of THS and THS-BSA on genomic DNA content of rat seminal vesicles differed dramatically. At a normal physiological plasma concentration of Tes in the rat (7.72 nM), THS increased the DNA content of
whereas THS-BSA had no significant effect ($P > 0.05$); the common superscript are significantly different (0.0085).

Precontracted with PE, PGF 2α, or K+ channels in the endothelium-denuded rat thoracic aortas.

Relaxation responses to 50 μM testosterone in paired, endothelium-denuded male rat thoracic aortas precontracted with PE, PGF 2α, or KCl.

Table 2. Relaxation responses to 50 μM testosterone in endothelium-denuded male rat thoracic aortas precontracted with PE, PGF 2α, or KCl 📖

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration, ng/ml</th>
<th>DNA Content, ng/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (MEM)</td>
<td>71.9 ± 21.6a</td>
<td></td>
</tr>
<tr>
<td>Testosterone hemisuccinate</td>
<td>3 170 ± 26.8b</td>
<td></td>
</tr>
<tr>
<td>(ester analog)</td>
<td>30 188 ± 4.8b</td>
<td></td>
</tr>
<tr>
<td>Testosterone hemisuccinate-BSA</td>
<td>3 87.5 ± 4.8*</td>
<td></td>
</tr>
<tr>
<td>(BSA conjugate)</td>
<td>30 92.5 ± 7.5*</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>3 87.5 ± 20.6*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± standard error ($n = 4$ rats). *Mean values for DNA content without common superscript are significantly different (0.0025 $P < 0.0001$). Rat seminal vesicles were sectioned and then incubated in MEM for 48 h, either alone (control) or in the presence of testosterone hemisuccinate (3 or 30 ng/ml), testosterone hemisuccinate-BSA (3 or 30 ng/ml), or BSA (3 ng/ml). Nucleic acids were extracted using Trizol reagent, and DNA was quantified spectrophotometrically at 260 nm. Nucleic acid contents were normalized by tissue wet weight.

DISCUSSION

In the present investigation, the structural specificity of Tes-induced vasorelaxation and the role of VSM K+ channels in this nongenomic effect of Tes were examined in the rat thoracic aorta. The results reveal that Tes-induced vasorelaxation is a structurally specific effect of the androgen molecule that is enhanced in more polar analogs that have a lower permeability to the VSM cell membrane and that the Endo-independent vasorelaxing effect of Tes involves activation of K+ efflux through K+ channels in VSM, perhaps via the KV.

The results of the Tes analog and metabolite experiments in the present study demonstrate that Tes-induced vasorelaxation is a structurally specific effect of the androgen molecule, which exhibits a structure-function relationship fundamentally different from that of its well-known genomic effects in reproductive tract target tissues. Of particular interest is the substantial vasorelaxation efficacy of the major nonpolar excretory metabolites And and Etio, which are virtually devoid of genomic effects in reproductive tissues, and the significantly lower vasorelaxation efficacy and potency of DHT, which exhibits at least twofold greater efficacy than Tes in its genomic effects in reproductive tissues (25, 32). Similarly, the esterified Tes analogs TEN and THS, which exert potent, long-acting genomic effects in vivo (28), exhibited among the lowest vasodilatory efficacies of all of the analogs studied. The structurally specific vascular effects of Tes are present in both its Endo-dependent and -independent vasorelaxing effects in the rat aorta. Because previous studies suggest that the Endo-dependent component of Tes-induced vasorelaxation is mediated primarily through nitric oxide (7), these findings suggest that Tes exerts minor but structurally specific nongenomic effects on the Endo as well as VSM of the rat aorta.

In the present study, Tes-induced relaxation of rat aortic VSM was inhibited substantially by 4-AP but not by apamin, TEA, or Gly. These findings suggest that...
Tes-induced vasorelaxation is mediated, at least in part, through activation of K⁺ efflux through K⁺ channels in VSM, perhaps via the Kv, resulting in hypopolarization and relaxation of aortic VSM. In contrast, the failure of apamin, TEA, or Gly to alter the acute vascular responses to Tes suggests that neither KCa nor KATP channels are involved in Tes-induced vasorelaxation. Although these compounds are not entirely specific for these K⁺ channels, at the concentrations used in these studies, these blockers are relatively selective for the KCa, KATP, and Kv channels, respectively. A role for K⁺ channels in Tes-induced vasorelaxation is further supported by the results of the high extracellular K⁺ experiments, which demonstrated that the vasodilatory effect of Tes at the most sensitive part of its concentration response (i.e., near EC₅₀) was attenuated substantially in aortas precontracted with 80 mM KCl, compared with those precontracted with either PE or PGF₄α.

The results of the present study confirm and extend the limited findings available on the direct effects of Tes on the vasculature. The initial studies of the rat aorta established that Tes-induced vasorelaxation is gender and androgen receptor independent and involves both Endo-dependent (nitric oxide) and -independent mechanisms of action (7). Similar Endo-dependent (nitric oxide) and -independent vasodilatory effects of Tes in canine coronary conductance and resistance arteries in vivo were reported by Chou et al. (4). Gly attenuated the vasodilatory effect of Tes by about one-third in coronary resistance vessels but not in epicardial conductance vessels, suggesting that KATP channels are involved in Tes-induced vasorelaxation. In contrast, Yue et al. (44) demonstrated an acute vasodilatory effect of Tes in the rabbit aorta and coronary artery in vitro that was Endo independent and, in part, appeared to involve a Tes-mediated increase in K⁺ conductance. In more recent studies of the Wistar-Kyoto and spontaneously hypertensive rat (SHR) aortas, Honda et al. (16) reported that Tes-induced vasorelaxation involved activation of K⁺ channels and was partially Endo dependent, similar to the findings of the present study; however, both KATP and Kv channels appeared to mediate the effects of Tes in Wistar-Kyoto and SHR aortas, and the role of the Kv channel was enhanced in the SHR aorta. In recent studies of the porcine coronary artery, Crews and Khalil (8) reported that Tes-induced vasorelaxation resulted from inhibition of extracellular Ca²⁺ entry (⁴⁵Ca²⁺ influx) in addition to other mechanisms that appeared sensitive to extracellular K⁺ concentration (i.e., K⁺ channel activation). Unequivocal evidence that K⁺ channels are the primary mediator of the acute vasodilatory effects of Tes was recently established by Deenadayalu et al. (10). Patch-clamp experiments on isolated coronary VSM cells revealed that the effect of Tes was mediated through the selective activation of the large-conductance KCa. Only one recent report exists of an acute, presumably nongenomic vasoconstrictor effect of Tes (3). Taken together, these findings provide broad evidence that the acute vasodilatory effect of Tes involves, at least in part, activation of VSM K⁺ channels. Although several different K⁺ channels have been implicated or identified, this likely reflects regional vascular and/or species differences in the expression of VSM K⁺ channels involved in the regulation of vascular tone.

Several recent studies have documented direct vasodilatory effects of estrogen on the vasculature involving rapid, presumably nongenomic mechanisms of action. Similar to the effects of Tes, the rapid vasodilatory effects of estrogen occur within minutes and have been attributed to endothelial (6, 12, 43) and/or VSM mechanisms (6, 12, 19, 27, 35). Several studies have demonstrated that the Endo-independent vasorelaxing effect of estrogen involves inhibition of VSM voltage-operated Ca²⁺ channels (19, 35, 45), although estrogen-induced increases in VSM large-conductance KCa channel activity have also been reported (42). Thus the acute vasodilatory effects of both estrogen and Tes appear to involve highly specific but different effects on the VSM cell membrane.

The unique structure-function relationship of the Endo-independent vasorelaxing effect of Tes established in the present study and the evidence that this effect involves activation of K⁺ efflux through K⁺ channels in VSM, perhaps via the Kv, strongly suggest that this nongenomic vascular effect of Tes involves a structurally specific interaction with the VSM cell membrane. Although neither membrane potential nor K⁺ channel activity was directly measured in this study, the data do suggest that Tes interacts at or near K⁺ channels and perhaps other membrane signal transduction proteins. A similar structure-function relationship has been reported for the nongenomic effects of androgen analogs on a thyroid hormone-sensitive Ca²⁺-ATPase system present in the cell membrane of the rabbit reticulocyte (22). In that study, as in the present study, the 5α-androstane analogs (such as DHT) exhibited substantially less effect than Tes on Ca²⁺-ATPase activity, whereas the 5β-androstanes (such Etio) had nearly the same effect as Tes, leading the investigators to conclude that a structurally specific interaction between Tes and thyroid hormone occurred in the reticulocyte cell membrane at or near the Ca²⁺-ATPase system.

Although the previous analog studies of Lawrence et al. (22) and Yue et al. (44) demonstrated that spatial conformation of the steroid molecule (i.e., angular vs. flat) is an important determinant of the nongenomic efficacy of the androgen analogs, the reason for this structural relationship is uncertain. In contrast, the results of the Tes analog experiments in the present study establish that the relative polarity of the androgen molecule is a primary determinant of its vasorelaxation efficacy and/or potency. The esterified Tes analogs TEN and THS, which are substantially less polar (and, therefore, more lipid soluble) than Tes, exhibited significantly lower efficacy and potency for vasorelaxation than did Tes. The polarity of the esterified analogs is inversely proportional to the molecular weight of their ester group (28); thus TEN, which
contains a larger ester group than THS, is less polar and exhibits dramatically lower vasorelaxation efficacy and potency than THS. These data demonstrate that polarity and, therefore, lipid solubility of the androgen molecule are important determinants of vasorelaxation efficacy and potency; thus it is proposed that Tes-induced vasorelaxation is a structurally specific effect of the Tes molecule, which is enhanced in more polar analogs that have a lower permeability to the VSM cell membrane. This idea is supported by results of the THS-BSA experiments, which demonstrated that conjugation of the nonpolar esterified analog THS to BSA, which presumably eliminates the permeability of this analog to the VSM cell membrane, dramatically enhanced both the efficacy and potency of this analog to cause vasorelaxation. The results of the experiment on the genomic effects of THS demonstrate, unequivocally, that conjugation of THS to BSA eliminates the permeability of this nonpolar analog to the cell membrane and, thereby, its ability to enter the cell and exert genomic effects.

The cellular mechanism underlying the effect of Tes to activate VSM K\(^+\) efflux through K\(^+\) channels and cause vasorelaxation of the rat aorta is uncertain. The results of past and present androgen analog studies in the rabbit reticulocyte and in rat aortic VSM strongly suggest that the effect of Tes involves a structurally specific interaction with the VSM cell membrane at or near K\(^+\) channels and perhaps other membrane signal transduction proteins. Similarly, a variety of studies have demonstrated that rapid, nongenomic effects of glucocorticoid, mineralocorticoid, and ovarian sex steroid hormones in neural, renal, and reproductive tissues are structurally specific effects of these molecules, which involve interactions with cell membrane structures, including ion channels, ion transporters, and hormone plus neurotransmitter receptors (26, 41). However, several other explanations are possible. For example, membrane binding sites for glucocorticoid, mineralocorticoid, and ovarian sex steroid hormones have been identified in a variety of neural, renal, and reproductive tissues, and receptor-mediated formation of intracellular second messengers, such as cyclic nucleotides or inositol 1,4,5-triphosphate, or increases in cytosolic Ca\(^{2+}\) have been reported (26, 41). Two recent studies of the nongenomic effects of androgens in nonvascular tissues lend strong support to the idea that the vasorelaxing effects of Tes involve interaction with specific structure(s) in the VSM cell membrane. Górczynska and Handelsman (13) reported that Tes produced a rapid and specific increase in intracellular Ca\(^{2+}\) in rat Sertoli cells and that this effect persisted when Tes was conjugated to BSA to prevent cellular entry by the hormone. More recently, Benton et al. (2) identified binding sites for Tes on the mouse splenic T-cell membrane and that Tes-BSA conjugate induced a rapid rise in cytosolic Ca\(^{2+}\) via nonvoltage-gated Ca\(^{2+}\) channels. Alternatively, steroid hormones may stimulate formation of second messengers through direct effects on their catalytic regulatory enzymes. For example, the Endo-independent vasorelaxing effect of 17β-estradiol on porcine coronary artery VSM cells is mediated through steroidal activation of the nitric oxide-cyclic GMP pathway (9). Thus Tes may activate VSM K\(^+\) channels via direct structural interaction or through membrane receptor-dependent or -independent formation of an intracellular second messenger.

A variety of clinical and epidemiological studies have established that marked sexual dimorphism exists in a variety of human cardiovascular diseases. The well-established observations that hypertension and coronary artery disease occur less frequently in premenopausal women than in men (24, 39) have led to the concept that estrogen has beneficial, whereas Tes has deleterious, effects on the heart and vasculature. Although numerous, recent studies in both animal and human vascular preparations have demonstrated rapid vasorelaxing effects of estrogen, few studies have investigated the direct vascular actions of Tes, despite several early clinical reports on the antianginal effects of Tes. These antianginal effects were ascribed to a direct vasodilatory effect of Tes on the human coronary vasculature (11, 14, 23, 40), which has been confirmed in more recent studies (30, 31). The results of the present study establish that the Endo-independent vasorelaxing effect of Tes in the rat aorta results from a structurally specific interaction with the VSM cell membrane, perhaps at or near K\(^+\) channels, which is enhanced in more polar analogs that have a lower permeability to the VSM cell membrane. The ability of Tes to acutely regulate vascular tone may explain, at least in part, the early clinical reports on the beneficial effects of Tes on angina and myocardial ischemia. Thus the development of nonpermeable androgen analogs, which lack androgenic activity at the genomic level, may provide therapeutic potential for the regulation of coronary and/or systemic vascular tone, although further studies will be necessary to fully understand the acute nongenomic effects of Tes on the vasculature.

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