Testosterone Increases Thromboxane A\textsubscript{2} Receptors in Cultured Rat Aortic Smooth Muscle Cells

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Previous studies have demonstrated increased contractile responses to thromboxane A\textsubscript{2} (TXA\textsubscript{2}) mimetics in aortas obtained from male rats compared with those obtained from females. This study was designed to determine the effects of testosterone and 17β-estradiol treatment on TXA\textsubscript{2} receptors in cultured rat aortic smooth muscle cells (RASMCs). TXA\textsubscript{2} receptor affinity and density were determined through equilibrium binding experiments using the TXA\textsubscript{2}/prostaglandin H\textsubscript{2} mimetic [1S-(1α,2β-(5Z),3α(1E,3R*)]-7-[3-(3-hydroxy-4-(4'-125Iiodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]heptan-2-yl]-5-heptenoic acid (125I-BOP). Incubation with testosterone (100 nM) for 24 or 48 hours resulted in a significant (p<0.05) 31% and 48% increase in TXA\textsubscript{2} receptor density without any change in affinity. 17β-Estradiol (100 nM) had no significant effect on either the density or affinity of TXA\textsubscript{2} receptors. Coincubation with the testosterone receptor antagonist hydroxylflutamide (1 μM) blocked the testosterone-induced increase in TXA\textsubscript{2} receptor density. The maximum increase in intracellular free calcium induced by I-BOP was significantly (p<0.05) greater in testosterone-treated RASMCs than controls. Similarly, increases in inositol trisphosphate induced by the TXA\textsubscript{2}/prostaglandin H\textsubscript{2} mimetic U46619 were significantly (p<0.05) greater in testosterone-treated RASMCs compared with controls. The results demonstrate that testosterone increases vascular TXA\textsubscript{2} receptor density and support the notion that sex steroid hormones modulate the expression of this receptor. (Circulation Research 1991;69:638–643)

Thromboxane A\textsubscript{2} (TXA\textsubscript{2}) and its immediate precursor prostaglandin H\textsubscript{2} (PGH\textsubscript{2}) are potent vasoconstrictor and proaggregatory substances synthesized in large quantities by platelets. Because of their common pharmacological effects, they are believed to share a common receptor, known as the TXA\textsubscript{2}/PGH\textsubscript{2} receptor.\textsuperscript{1} TXA\textsubscript{2} synthesis is increased in a variety of cardiovascular diseases, and it has been implicated as an important pathophysiological mediator in these diseases.\textsuperscript{2–4} In this regard, the number of TXA\textsubscript{2} receptors in human platelets is increased during acute myocardial infarction in patients and returns to normal values during the convalescent period.\textsuperscript{5}

Before menopause, there is an increased incidence of acute myocardial infarction.\textsuperscript{6–8} These observations have raised the possibility that androgenic steroids may play a role in the pathogenesis of cardiovascular diseases.

Piper and Vane\textsuperscript{9} originally reported that aortas obtained from male rabbits were more sensitive to “rabbit aorta contracting” substance, a mixture of PGH\textsubscript{2} and TXA\textsubscript{2}, than those obtained from females. Penhos et al\textsuperscript{10} and Myers et al\textsuperscript{11} reported that there were sex differences in the sensitivity of mice to the lethal effects of arachidonic acid injected intravenously. In this model of thrombosis and pulmonary vasoconstriction, TXA\textsubscript{2} synthesis inhibitors and receptor antagonists protect against the lethal effects of arachidonic acid. Male mice were more sensitive than female mice, and administration of testosterone enhanced the sensitivity to the lethal effects of arachidonic acid. Karanian et al\textsuperscript{12} found that male rat aortas were more sensitive to the TXA\textsubscript{2} mimetic U46619 than female aortas and that the maximum response was greater in the males compared with the females. Sintetos et al\textsuperscript{13} also found that male rabbit aortas were more sensitive to U46619 than female aortas and that treatment of gonadectomized rabbits

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with testosterone increased this sensitivity. Collectively, these observations support the notion that the sex steroids may influence vascular TXA₂ receptors.

We conducted this study to determine the effects of testosterone and 17β-estradiol 1) on the affinity and density of TXA₂ receptors in cultured rat aortic smooth muscle cells (RASMCs) as determined using the radiolabeled TXA₂ agonist [1S-((α₂β₂₅z₂),3α (1E,3R²),4α]-7-[3-(3-hydroxy-4-(4′,125iodophenox)-1-butenyl)-7-oxabicyclo[2.2.1]heptan-2-y]l-5-heptenoic acid ([125I-BOP]) and 2) on TXA₂ mimetic–stimulated increases in intracellular free Ca²⁺ concentrations and inositol trisphosphate (IP₃) formation.

Materials and Methods

Materials

1-BOP and [125I]-BOP were synthesized in our laboratory as previously described. L657925, a TXA₂ receptor antagonist, was obtained from Merck Frosst Canada Inc., Point Claire, Dorval, Canada, and U46619 from Upjohn Pharmaceutical Co., Kalnamoo, Mich. Testosterone and 17β-estradiol were obtained from Sigma Chemical Co., St. Louis, Mo., and hydroxyflutamide was provided by Schering Corp., Kenilworth, N.J. Fura-2 AM was purchased from Calbiochem Corp., La Jolla, Calif. [3H]Myo-inositol was purchased from New England Nuclear, Boston, Mass. Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), and antibiotic solution were purchased from Gibco, Grand Island, N.Y. All other chemicals were of reagent grade.

Culture of Rat Aortic Smooth Muscle Cells

RASMCs were cultured using a modification of the procedure of Ross. Briefly, male rats were anesthetized with sodium pentobarbital, and the aorta was removed. The aorta was opened longitudinally and dissected into 2–3-mm segments and placed in the side down on a Petri dish (100×15 mm, Falcon). These segments were incubated in 10 ml DMEM containing 10% FCS and 1% antibiotic/antimycotic solution (penicillin, streptomycin/fungizone, Gibco) at 37°C in a humidified atmosphere of 95% air–5% CO₂. The growth medium was changed every 3–4 days. On reaching confluence, cells were harvested using 3 ml 0.05% trypsin–EDTA. The suspension then was dispensed into T-150 flasks for further growth. Cells from passages 3–8 were used for these experiments.

Sex Steroid Hormone Treatment of Cultured Cells

Confluent cells were treated in DMEM containing vehicle (0.001% ethanol), or 100 nM testosterone or 17β-estradiol, or DMEM containing vehicle and 10% FCS for 24 hours or 1% FCS for 48 hours for the binding experiments. At the end of the latter experiments, the medium was exchanged. The testosterone concentration decreased by 46% (n = 13) after 24 hours of incubation with the cells (data not shown). There were no measurable levels of testosterone (<0.03 ng/ml) in the medium containing 1% or 10% FCS (data not shown). Hydroxyflutamide (1 μM), an androgen receptor antagonist, was used to antagonize the effect of testosterone. In a separate series of experiments, confluent cells were cultured in DMEM containing 10% FCS with either vehicle, 100 nM testosterone, 1 μM hydroxyflutamide, or 100 nM testosterone plus 1 μM hydroxyflutamide for 24 hours. Because of the variance in binding capacities among cell lines, cells for each treatment group were prepared from the flasks of cells of the same line and passage on the same occasion. Treatment of the cells with testosterone and 17β-estradiol had no significant effect on cell growth as assessed by cell count and total protein per flask (data not shown).

Radioligand Binding Assays

Cells were washed twice with phosphate buffered saline (37°C) containing 10 μM indomethacin and were harvested into 30 ml DMEM containing 10% FCS using 3 ml of 0.05% trypsin–EDTA. After centrifugation at 100g for 5 minutes, cells were washed with 20 ml Hank’s balanced salt solution (HBSS) (mM) NaCl 135, KCl 5, Na₂HPO₄ 0.4, KH₂PO₄ 0.45, MgSO₄ 0.8, NaHCO₃ 4, CaCl₂ 1, glucose 5.5) and were centrifuged at 100g for 5 minutes. The pellets were resuspended in HBSS to the concentration of 5×10⁶ cells/ml. The radioligand binding assay was performed by incubating cells (1×10⁶ per tube) with approximately 2×10⁶ cpm (~0 pM) of [125I]-BOP and [125I]-BOP in a final volume of 400 μl HBSS at 37°C. The reaction was stopped by adding 4 ml ice-cold buffer, and the mixture was filtered through GF/C glass fiber filters (Whatman Inc., Clifton, N.J.) and washed three times with 4 ml ice-cold buffer within 10 seconds. Nonspecific binding was defined as the amount of bound radioactivity in the presence of L657925 (10 μM), a stereoselective TXA₂ receptor antagonist. Kᵅ and Bₘₐₓ values for I-BOP were determined using the LIGAND computer program. The Bₘₐₓ values were expressed as both the number of receptors per cell and also the number of femtomoles per milligram protein.

Intracellular Free Calcium Concentrations

Confluent cells were incubated in DMEM containing 10% FCS with vehicle, 100 nM testosterone, or 100 nM 17β-estradiol for 24 hours before harvesting. RASMCs harvested by trypsin were resuspended into HEPES buffer ([mM] HEPES 10, NaCl 145, KCl 5.4, glucose 5.5) without Ca²⁺ and Mg²⁺ and were incubated with 8 μM fura 2-AM for 1 hour. After two washes with the buffer, cells were resuspended to 2×10⁶ cells/ml in HEPES buffer containing 1 mM Ca²⁺ and 1 mM Mg²⁺. The cells were placed in quartz cuvettes at 37°C with continuous stirring. Basal and I-BOP–stimulated increases in intracellular free Ca²⁺ concentrations were measured using a fluorescence spectrophotometer with excitation and emission wavelengths of 340 and 500 nm, respectively. Calibration was done by lysing the cells with 10 mM digitonin.
to determine maximum fluorescence (Fmax) and then 10 mM EGTA and 20 mM Tris base (pH 8.0) were added to determine minimum fluorescence (Fmin). For both control and sex steroid–treated cells, a full concentration–response study was performed.

Measurement of Inositol Trisphosphates

Cultured RASMCs were seeded in six-well plates. The confluent cells were incubated with 10 μCi (160 pmol) [3H]myoinositol in 2 ml DMEM containing 1% FCS with vehicle or testosterone (100 nM) for 48 hours. After incubation, cells were washed twice with HEPES buffer (1 mM NaCl 145, HEPES 20, KCl 5, NaHPO4, 1.2, MgSO4, 1.2, CaCl2 2, glucose 5) without LiCl and were stimulated with 1 μM U46619 in HEPES buffer with 10 mM LiCl for 1 minute. In preliminary studies, we have found this time point to be the time for maximal stimulation for the formation of IP3. The reaction was stopped by addition of 15% trichloroacetic acid; the wells then sat at 4°C for 30 minutes and were extracted using ethyl ether. The inositol phosphates were separated by anion-exchange column chromatography. Briefly, the radioactive phosphates extracted were separated from each other with 5 mM disodium tetraborate/60 mM sodium formate for glycerophosphoinositol, 0.1 M formic acid/0.2 M ammonium formate for inositol monophosphate (IP1), 0.1 M formic acid/0.4 M ammonium formate for inositol bisphosphate (IP2), and 0.1 M formic acid/1.0 M ammonium formate for IP3 as described by Berridge. The radioactivity in the IP3 fraction was measured with a liquid scintillation counter.

Data Analysis

We assessed differences between groups using a paired t test for the intracellular Ca2+ and IP3 experiments and the initial binding experiments (Figures 2 and 4). A one-way analysis of variance followed by a Fisher’s protected least-squares difference post hoc test was used to analyze the testosterone and testosterone plus hydroxyflutamide data (Figure 3). All data are expressed as mean±SEM.

Results

Radioligand Binding Assays

The binding of I-BOP, a TXA2 mimetic, to RASMCs has been characterized extensively. In the cells treated with or without sex steroid hormones for 24 hours (Figure 1), I-BOP bound to the TXA2/PGH2 receptor on RASMCs in a saturable manner. Scatchard analysis revealed a single class of TXA2 receptors on RASMCs in all three groups. RASMCs treated with 100 nM testosterone or 100 nM 17β-estradiol for 24 hours showed no significant difference in Kd values (342±38, 358±32, and 355±26 pM for control, testosterone-treated [Figure 2a], and 17β-estradiol–treated cells, respectively, n=6). Testosterone-treated cells showed significantly (p<0.05) higher Bmax values compared with control (16,208±1,430 sites/cell for testosterone-treated cells and 12,340±1,468 sites/cell for control, n=6) (Figures 1 and 2b). When Bmax was normalized to protein content, there was also a significantly greater Bmax.

**Figure 1.** Representative equilibrium binding analysis of I-BOP to control and testosterone-treated (100 nM for 24 hours) cells. Assay conditions are described in “Materials and Methods.” Scatchard analysis of saturation binding data is shown in inset. Kd and Bmax values are representative of five experiments. B/F, bound/free.
(64.4±4.3 fmol/mg protein for testosterone-treated cells and 51.4±5.6 fmol/mg protein for control, p<0.05, n=6) (Figure 2c). On the other hand, 17β-estradiol–treated cells showed no significant difference in Bmax values compared with the control group (13,247±2,716 sites/cell or 63.4±11.4 fmol/mg protein, n=6). We also determined if exposure to the steroids for a longer period of time (48 hours) and at a lower FCS concentration (1%) would produce a greater effect. In cells exposed to the sex steroids for 48 hours, there were also no significant differences in Kd values among the three groups (271±52, 297±40, and 257±27 pM for control, testosterone-treated, and 17β-estradiol–treated cells). Significantly higher Bmax values were obtained in testosterone-treated cells when compared with control cells (10,002±2,245 sites/cell or 64.3±10.6 fmol/mg protein for testosterone-treated cells and 6,754±1,792 sites/cell or 42.6±10.9 fmol/mg protein for control cells, p<0.05, n=7) (Figures 2b and 2c). Incubation with 17β-estradiol did not significantly change Bmax values compared with controls (7,159±1,477 sites/cell or 50.2±15.7 fmol/mg protein, n=5). Incubation with testosterone for 24 and 48 hours increased TXA2 receptor densities 31% and 48%, respectively. To determine if this effect of testosterone was mediated by its receptor, RASMCs were cocultured with the testosterone receptor antagonist hydroxyflutamide. Treatment with testosterone significantly increased Bmax values (13,165±1,176 sites/cell or 76.3±3.0 fmol/mg protein, p<0.05 compared with control: 9,782±1,321 sites/cell or 57.4±2.9 fmol/mg protein, n=7) (Figure 3). Hydroxyflutamide completely antagonized the effect of testosterone to increase the Bmax values in the testosterone plus hydroxyflutamide group (9,942±908 sites/cell or 58.1±5.3 fmol/mg protein, p<0.05 versus testosterone group). These Bmax values also were not significantly different from those of control. Hydroxyflutamide itself had no effect on the Bmax value (9,930±757 sites/cell or 61.9±2.0 fmol/mg protein). No significant difference in Kd values was observed among the four groups (Figure 3).

**Intracellular Free Calcium Concentrations**

Stimulation of vascular smooth muscle cell TXA2 receptors has been shown to result in increases in intracellular free calcium.21 We have found that I-BOP produces dose-dependent increases in intracellular free calcium concentrations ([Ca2+]i) in RASMCs (Masuda et al, unpublished observations). Because testosterone increased the number of TXA2 receptors, we determined if this also was associated with TXA2/PGH2 agonist–induced increases in intracellular messengers, such as [Ca2+]i, and IP3 formation. The assays were done in pairs with control and testosterone-treated cells or control and 17β-estradiol–treated cells, using cells from the same line and passage. Basal [Ca2+]i, was 49±3 nM in control cells, 58±9 nM in testosterone-treated cells, and 72±16 nM in 17β-estradiol–treated cells (NS). The EC50 values for I-BOP–induced increases in [Ca2+]i, were 34.8±5.9 and 37.2±10.5 nM for control and testosterone-treated groups, respectively (NS, n=5). The EC50 values for I-BOP–induced increases in [Ca2+]i, were 21.8±6.9 and 31.2±10.9 nM for control and 17β-estradiol–treated groups, respectively (NS, n=5). Testosterone-treated cells showed significantly higher I-BOP–induced increases in maximum [Ca2+]i compared with controls (15.8±2.9 and 10.2±1.5 nM for testosterone-treated and control, respectively; p<0.05, n=7) (Figure 4). However, no difference in the I-BOP–induced increase in [Ca2+]i, was observed between 17β-estradiol–treated cells (14.5±5.0 nM) and control (20.3±3.5 nM, n=5). The increase in intracellular calcium elicited by I-BOP was antagonized by two different TXA2 antagonists, SQ-29,548 (1 μM) and L657925 (1 μM) (data not shown).

**Changes in Formation of Inositol Trisphosphate**

Basal levels of IP3 were 956±95 cpm/mg protein and 982±115 cpm/mg protein in control and testosterone-treated cells, respectively (n=5, NS). The
U46619 (1 μM)-induced increase in IP$_3$ was 261 ± 78 cpm/mg in testosterone-treated cells and was significantly higher than that of controls (142 ± 49 cpm/mg protein; p < 0.05, n = 5) (Figure 5). This IP$_3$ response to U46619 was antagonized by L657925 (1 μM).

**Discussion**

This study provides evidence that testosterone can directly modulate the number of functional TXA$_2$ receptors in cultured vascular smooth muscle cells. This effect is mediated by a testosterone receptor, because hydroxyflutamide, a testosterone receptor antagonist, blocked the increase. The increase in TXA$_2$ receptors induced by testosterone did not appear to be a result of a nonspecific anabolic action, because the amount of protein per cell and the amount of protein and cells per flask were not significantly different compared with the vehicle-treated group (data not shown). In addition, normalizing the TXA$_2$ receptor density per milligram of protein or sites per cell resulted in significantly greater values in the testosterone-treated group compared with controls. In contrast to testosterone, 17β-estradiol had no significant effect on TXA$_2$ receptor number or TXA$_2$ agonist-induced increases in [Ca$^{2+}$]. The effect of 17β-estradiol on TXA$_2$ receptors was, however, variable, unlike the consistent response to testosterone. The reason for the greater variability is uncertain but may simply reflect a lack of a significant effect of 17β-estradiol coupled with the variability in TXA$_2$ receptor density seen in these cells. Based on the previous observations that female rats or rats treated with 17β-estradiol were less sensitive to TXA$_2$ mimetics compared with males or testosterone treatment, it may have been predicted that 17β-estradiol might decrease the number of TXA$_2$ receptors. Aortas from male rats were used in these studies, and perhaps if aortas from female rats had been used, an effect of 17β-estradiol may have been seen. The failure of 17β-estradiol to have an effect is not due to a lack of receptors for it within vascular smooth muscle cells, because previous studies have shown that β-adrenergic receptors in cultured RASMCs are increased by treatment with 17β-estradiol. The possibility exists that 17β-estradiol does not decrease vascular TXA$_2$ receptors or that the rate of turnover of vascular TXA$_2$ receptors is too low for a significant reduction to occur within 24–48 hours. That a reduction in vascular receptors may occur in vivo is borne out by the observations of Sessa et al, who recently reported that dexamethasone administered to rabbits for 1 week resulted in a significantly decreased contractile response to TXA$_2$ mimetics and an associated decrease in TXA$_2$ receptors in aortic membranes. From their study, it is not known if this was a direct effect of dexamethasone to decrease TXA$_2$ receptors or was indirect through some other factors.

The testosterone concentrations used in this study are higher than the normal circulating levels of free testosterone but are achievable with pharmacological replacement doses and certainly with androgen abuse. These concentrations have been used previously in other in vitro studies of the effects of testosterone on cultured nonvascular smooth muscle cell receptors and do not necessarily reflect the free concentration of testosterone because of the presence of serum. Where this concentration would fall on a concentration–response curve currently is not known.

That the increase was in functional receptors is supported by the observations that TXA$_2$ agonist-induced changes in intracellular free calcium and IP$_3$ formation also were significantly increased by testosterone. Testosterone pretreatment significantly enhanced the maximum increase in [Ca$^{2+}$] induced by I-BOP. The EC$_{50}$ values for I-BOP–induced increases in [Ca$^{2+}$], were not significantly different in the testosterone-treated group compared with the vehicle-treated group. This would be expected, because the apparent affinity of the TXA$_2$ receptor was not changed by testosterone. U46619, another TXA$_2$ agonist, used at its maximum concentration also elicited significantly more IP$_3$ formation in testosterone-treated cells compared with control cells. Along with the lack of an effect of 17β-estradiol on TXA$_2$ receptors, U46619 also had no effect on the maximum increase in [Ca$^{2+}$], elicited by I-BOP. Collectively, these results indicate that testosterone increased the maximum response to TXA$_2$ in cultured RASMCs through an increase in receptor number without changing its affinity. The significance of the small increases in the [Ca$^{2+}$], and IP$_3$ formation in the testosterone-treated cells as they relate to the ultimate effect of testosterone to augment responsive-ness to TXA$_2$ remains uncertain.

Previously, it has been well established that there are sex-related differences in cardiovascular morbidity and mortality. In men, the incidence of heart attacks is much greater than in women before menopause. However, after menopause, this difference disappears. The reasons for the differences in cardiovascular morbidity and mortality between men and women remain unknown. However, the gonadal sex steroids have been implicated as etiologic factors. Furthermore, several reports of premature cardiovascular or cerebrovascular accidents in young ath-
letes with androgenic steroid abuse\textsuperscript{28–31} and a hypogonadal patient treated with testosterone\textsuperscript{26} suggest that androgenic steroids may play an important role in platelet hyperaggregability, vasospasm, and thrombotic cardiovascular diseases.

The relevance of the observations made in this study to the sex differences in cardiovascular diseases and androgen steroid–induced thrombotic cardiovascular diseases remains uncertain. Given the potential involvement of \( \text{TXA}_2 \) in the pathogenesis of these cardiovascular events, alterations in vascular \( \text{TXA}_2 \) receptors induced by androgens could be an important contributing factor. Clearly, these observations merit further investigation.

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References


\textbf{Key Words} • testosterone • 17\beta-estradiol • hydroxyflutamide • radioligand binding • thromboxane \( \text{A}_2 \) receptors
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