Flutamide Induces Relaxation in Large and Small Blood Vessels

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Hypothesis: Flutamide, a testosterone receptor antagonist, produces various beneficial effects in male rats following hemorrhagic shock, possibly as a result of a direct vasodilating effect on large and small vessels in the rat.

Design, Interventions, and Main Outcome Measures: The aorta and the small intestine were isolated from normal male and female Sprague-Dawley rats. Isolated aortic rings were placed in the organ bath and constricted by 2 × 10⁻⁷M norepinephrine bitartrate (Sigma, St Louis, Mo). Flutamide-induced and testosterone-induced vascular relaxation dose-response curves were then determined. The dose-response curves of flutamide were also determined in the small blood vessels of the isolated small intestine under conditions of constant flow following preconstriction induced by 5 × 10⁻⁶M norepinephrine bitartrate. The effects of prior incubation with testosterone (8 × 10⁻⁵M) and sex differences on flutamide-induced vascular relaxation were also examined in aortic rings and in the small intestine. Moreover, flutamide-induced relaxation in endothelium-denuded aortic rings and in aortic rings from animals subjected to trauma and hemorrhagic shock was examined.

Results: Flutamide induced significant relaxation in aortic rings and small intestinal blood vessels in healthy males. The flutamide-induced relaxation in vessels from normal males was partially attenuated by prior incubation with the male sex steroid testosterone, and was significantly lower in females. Flutamide-induced vascular relaxation in the aorta was partially attenuated by endothelium removal, but it was not significantly affected by trauma and hemorrhagic shock in male rats.

Conclusions: Flutamide has a direct vasodilating effect on large and small vessels in rats, which involves sex-dependent mechanisms. Thus, the beneficial effects of flutamide on cardiovascular responses in males following trauma and hemorrhagic shock may be due to the direct vascular relaxation induced by this agent.

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Flutamide, a nonsteroidal testosterone receptor antagonist, has been shown to restore depressed immune function in male mice following trauma and hemorrhage.¹ ² With regard to organ function, testosterone receptor blockade following trauma or hemorrhage has been shown to improve the depressed cardiac, hepatic, adrenal, and endothelial functions in male rats.³-⁵ However, the precise mechanism of the salutary effect of flutamide remains unknown. Previous studies have demonstrated that hypertension and coronary artery disease occur more frequently in men than in age-matched premenopausal women.⁶ Some reports indicate that both the therapeutic use of androgenic steroids and their abuse by athletes are associated with the early onset of cardiovascular and cerebrovascular disease.⁷ ⁸ Experimental studies further support the finding that estradiol-treated rats have significantly lower total collagen, a lower collagen-elastin ratio, and higher percentages of elastin in vascular connective tissue than those rats receiving testosterone.¹⁰ Furthermore, systolic blood pressure was highest in rats receiving testosterone and lowest in rats receiving estradiol.¹⁰ Several recent studies¹¹-¹⁵ have also demonstrated in vitro the rapid vasodilating effects of estrogen at physiological and pharmacological doses in a variety of animal (eg, canine, rabbit, and rat) and human vascular preparations. Moreover, the rapid vasodilatory effects of estrogen have also been observed in vivo in both nonhuman primates and humans.¹⁵ ¹⁶

Although studies have reported direct effects of estrogen on the vasculature, there is no information available to indicate whether flutamide has any direct effects on the vasculature. We hy-
pothesized that flutamide directly induces vascular relaxation in large and small blood vessels, and this effect on the vasculature may explain its beneficial effects on organ functions following hemorrhagic shock. The aim of this study, therefore, was to determine whether flutamide directly affects the vascular tone in isolated vessel rings and isolated perfused gut preparations. Moreover, the effects of sex and testosterone on the vascular activity induced by flutamide were examined.

MATERIALS AND METHODS

ANIMALS

Age-matched adult male and female Harlan Sprague-Dawley rats (weight, 275-325 g and 200-250 g, respectively) were studied. All female rats were studied at the proestrus stage of the reproductive cycle, as defined by the presence of both leukocytes and nucleated epithelial cells in approximately equal numbers on vaginal smears. Animals were fasted 16 hours before the experiment but were allowed water ad libitum. This project was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

AORTIC RING PREPARATION AND DETERMINATION OF FLUTAMIDE-INDUCED AND TESTOSTERONE-INDUCED VASCULAR RELAXATION

Immediately after the death of the animals by an overdose of ether, the rat chests were opened and thoracic aortas were rapidly removed. The isolated thoracic aorta was immediately immersed in Krebs-Ringer bicarbonate (HCO₃) solution (composition: 118.3mM sodium chloride, 4.7mM potassium chloride, 2.5mM calcium chloride, 1.2mM magnesium sulfate, 1.2mM potassium phosphate monobasic, 25.0mM sodium bicarbonate, 0.026mM Ca EDTA, and 11.1mM glucose), which was aerated with a mixture of 95% oxygen and 5% carbon dioxide (pH, 7.4; PO₂, 580 mm Hg). The thoracic aorta was trimmed with care to prevent any damage to vascular endothelial cells and was cut into rings approximately 2.5 mm in length. The aortic ring was carefully mounted on 2 specimen holders and placed in a glass organ chamber containing 20 mL of aerated Krebs-Ringer HCO₃ solution at 37°C. One holder was stationary while the other was connected to an isometric force-displacement transducer (model FT03; Grass Instruments, Quincy, Mass) coupled to a polygraph (model 7D; Grass Instruments). The aortic ring was incubated for 60 minutes at a tension of 1000 mg, during which time the organ chamber was rinsed every 15 minutes with the aerated Krebs-Ringer HCO₃ buffer. When basal tension was stable, a submaximal contraction (approximately 75% of maximal contraction) was induced by 2×10⁻⁶M nor-epinephrine bitartrate (Sigma-Aldrich Corp, St Louis, Mo). The nonsteroidal testosterone receptor antagonist, flutamide (Schering-Plough Corp, Kenilworth, NJ; concentration range, 10⁻⁰M to 16×10⁻⁰M), or an equal volume of propanediol, the nontoxic flutamide vehicle, was applied cumulatively thereafter with or without prior incubation with testosterone (Sigma-Aldrich Corp). Incubation with testosterone (8×10⁻⁶M) was begun 13 minutes before nor-epinephrine-induced ring contraction and was maintained after each wash. After each series of agent additions, the ring preparations were washed with Krebs-Ringer HCO₃ solution and allowed to reequilibrate for at least 30 minutes. Additionally, a dose-response curve to testosterone (concentration range, 10⁻⁰M to 10⁻⁴M) with or without preincubation with flutamide (10⁻⁰M) was performed. Flutamide-induced vascular relaxation was tested in both male and female rats to assess sex-based differences. Furthermore, flutamide-induced vasodilation was determined in male aortic rings subjected to endothelial denudation. Flutamide-induced relaxation was also examined in aortic rings from male rats at 1.5 hours after trauma-hemorrhage and resuscitation. We have previously described this model of trauma-hemorrhage.

ISOLATED SMALL INTESTINE AND DETERMINATION OF FLUTAMIDE-INDUCED SMALL VESSEL RELAXATION

In additional animals, the right femoral vein was cannulated under isoflurane (1.5%, balanced with air) anesthesia, and the anesthesia was maintained by injection of pentobarbital sodium (30 mg/kg of body weight) via the femoral catheter. Immediately after intravenous injection of 0.5 mL of heparin solution (500 U), the small intestine was isolated without removal from the abdominal cavity. The superior mesenteric artery and the portal vein were cannulated with PE-50 and PE-90 tubing, respectively. The branches of blood vessels to and from the cecum, and the ascending and transverse portions of the colon were then ligated. While the rat was still alive, the isolated intestine was perfused with aerated Krebs-Ringer HCO₃ buffer (a mixture of 95% oxygen and 5% carbon dioxide at 37°C) through the superior mesenteric artery catheter (PE-50). Dextran 70 (6%; Sigma-Aldrich Corp) was added to the Krebs-Ringer HCO₃ buffer to prevent intestinal edema. After 30 minutes of equilibration at a constant perfusion rate of 5 mL per minute, mean±SE perfusion pressure was 28.5±1.5 mm Hg. Blood vessels in the isolated intestine were then contracted with 5×10⁻⁶M norepinephrine bitartrate, which increased the mean±SE perfusion pressure to 113.7±6.1 mm Hg. The vascular responses to flutamide (range, 0.5×10⁻⁶M to 8×10⁻⁶M) with and without prior incubation with testosterone (8×10⁻⁶M) were measured thereafter by determining the changes in perfusion pressure in the isolated small intestine. The intestinal preparations were viable throughout the study period, as determined by the similar rise in the norepinephrine-induced increase in perfusion pressure at the end of the experiments vs that at the beginning of the experiments. The vascular responses to flutamide in isolated intestines of male and female rats were compared. It has been reported that resistance blood vessels (ie, small arteries and arterioles), rather than large arteries, play a major role (90%) in maintaining peripheral resistance. Therefore, changes in perfusion pressure in isolated small intestinal preparations reflect the reactivity of small arteries and arterioles in the intestine.

STATISTICAL ANALYSIS

The vascular relaxation responses were expressed as a percentage reduction of the initial vascular tension (ie, 2×10⁻⁶M norepinephrine-induced contraction in isolated thoracic aortic ring study and 5×10⁻⁶M norepinephrine-induced contraction in the isolated small intestine). Two aortic rings from each animal were studied, and the values were averaged. All data are presented as mean±SE. One-way analysis of variance (ANOVA) and Tukey test were employed for the comparison between groups, and the differences were considered significant at P<.05.

RESULTS

WEIGHT OF AORTIC RINGS AND ISOLATED INTESTINES, AND NOREPINEPHRINE-INDUCED INITIAL TENSION

Table 1 presents how the weight of aortic rings and the initial tension induced by 2×10⁻⁶M norepinephrine...
Table 1. Weight of Aortic Rings and Initial Tension Induced by 2 × 10⁻⁵ M Norepinephrine

<table>
<thead>
<tr>
<th></th>
<th>Weight, mg</th>
<th>Initial Tension, mg</th>
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<tbody>
<tr>
<td>Males</td>
<td>2.12 ± 0.12</td>
<td>991.8 ± 37.7</td>
</tr>
<tr>
<td>Males†</td>
<td>2.08 ± 0.20</td>
<td>930.3 ± 62.7</td>
</tr>
<tr>
<td>Females</td>
<td>2.05 ± 0.22</td>
<td>981.1 ± 55.0</td>
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</table>

*Data are presented as means ± SE (n = 6). Norepinephrine was in the bitartrate form.
†Males with or without testosterone incubation.

Table 2. Weight of Isolated Small Intestine and Perfusion Pressure Induced by 2 × 10⁻⁶ M Norepinephrine

<table>
<thead>
<tr>
<th></th>
<th>Intestinal Weight, g</th>
<th>Initial Pressure, mm Hg</th>
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<tbody>
<tr>
<td>Males</td>
<td>6.22 ± 0.20</td>
<td>113.6 ± 6.1</td>
</tr>
<tr>
<td>Females</td>
<td>6.07 ± 0.20</td>
<td>109.8 ± 8.7</td>
</tr>
</tbody>
</table>

*Data are presented as means ± SE (n = 6). Norepinephrine was in the bitartrate form.

Figure 1. Flutamide-induced vascular relaxation of the aorta and small intestinal vessels from males with or without testosterone. Cumulative dose responses to various concentrations of flutamide, flutamide with testosterone before incubation, and equal volume of propanediol as a vehicle in aortic rings (A) and isolated small intestinal vessels (B) of male rats. Values are expressed as percentages of vascular relaxation from initial vascular tension or pressure reduction from initial perfusion pressure induced by norepinephrine bitartrate. Data are presented as means ± SE (n = 6–7 per group) and were compared by 1-way analysis of variance and Tukey test. Asterisks indicate P < .05 vs vehicle; daggers, P < .05 vs flutamide alone.

EFFECT OF FLUTAMIDE ON LARGE AND SMALL VESSELS IN MALES

Figure 1 indicates the response of aortic rings and isolated small intestines to flutamide in male rats. As shown in Figure 1A, flutamide induced significant dose-dependent aortic vascular relaxation. The apparent threshold for flutamide-induced relaxation was 10⁻⁵ M flutamide (13.7% ± 5.7%), and maximal relaxation (93.4% ± 2.0%) was obtained with 16 × 10⁻⁵ M flutamide. 1,2-Propanediol (vehicle), however, did not significantly affect aortic vascular tension. Similar results were observed in isolated small intestinal perfusion (Figure 1B). The lowest concentration of flutamide that significantly reduced intestinal perfusion pressure (23.7% ± 7.1%) was 5 × 10⁻⁴ M, and maximal reduction (100.0% ± 1.35%) was obtained with 8 × 10⁻⁴ M flutamide. However, 1,2-propanediol did not significantly affect perfusion pressure.

EFFECT OF TESTOSTERONE ON FLUTAMIDE-INDUCED VASCULAR RELAXATION IN MALES

As shown in Figure 1A, the threshold for flutamide-induced aortic relaxation was 4 × 10⁻⁵ M with prior testosterone incubation, which was 4-fold higher than the threshold of 10⁻⁵ M for flutamide-induced aortic ring relaxation without prior testosterone incubation. Furthermore, in the middle-dose range of flutamide (8 × 10⁻⁵ M), flutamide-induced aortic ring relaxation in the testosterone incubation group was significantly lower than the relaxation in the group without testosterone incubation. However, at the high-dose range of flutamide (16 × 10⁻⁵ M), no statistically significant difference was observed between these 2 groups. Similar results were observed in isolated small intestinal perfusion in male rats. As shown in Figure 1B, flutamide-induced small vessel relaxation in male rats was significantly reduced by prior incubation with testosterone.

EFFECT OF TESTOSTERONE WITH OR WITHOUT FLUTAMIDE PREINCUBATION ON LARGE VESSELS IN MALE RATS

Testosterone induced a significant dose-dependent relaxation in concentrations greater than 10⁻⁶ M. A maximal relaxation (53.4% ± 13.2%) was obtained with 10⁻⁵ M flutamide (Figure 2). Prior incubation with flutamide did not significantly change testosterone-mediated aortic relaxation (Figure 2).

SEX DIFFERENCES IN FLUTAMIDE-INDUCED VASCULAR RELAXATION

The threshold for flutamide-induced aortic relaxation was 4 × 10⁻⁵ M in females (Figure 3A), which was 4-fold
higher than the threshold in males ($10^{-5}$M). Furthermore, flutamide-induced aortic ring relaxation at various concentrations in females was significantly lower than the relaxation achieved by comparable concentrations of flutamide in males. Similarly, flutamide-induced reductions in isolated small intestine perfusion pressure at $2 \times 10^{-5}$M, $4 \times 10^{-5}$M, and $8 \times 10^{-5}$M were significantly lower in females than in males (Figure 3B).

**EFFECTS OF ENDOTHELIUM DENUDATION AND TRAUMA OR HEMORRHAGIC SHOCK ON FLUTAMIDE-INDUCED VASCULAR RELAXATION IN MALE RATS**

Flutamide-induced vascular relaxation in endothelium-denuded aortic rings was partially attenuated compared with the rings having intact endothelium (Figure 4A). The entire response curve to flutamide was displaced to the right approximately 10% to 20% in aortic rings without endothelium compared with aortic rings with intact endothelium. However, data in this study show that the relaxation induced by flutamide was not significantly different in aortic rings from sham and from animals 1.5 hours after hemorrhagic shock and resuscitation (Figure 4B).

**COMMENT**

Our previous studies have shown that flutamide, a testosterone receptor antagonist, restores the depressed immune function in male mice following trauma and hemorrhagic shock.\(^1\)\(^,\)\(^2\) Flutamide treatment also prevents vasoconstriction by testosterone,\(^18\)\(^,\)\(^19\) and improves cardiac, hepatic, adrenal, and endothelial functions in male rats following trauma and hemorrhagic shock.\(^3\)\(^,\)\(^5\) Therefore, we hypothesized that testosterone receptor blockade by flutamide would relax vessels, and that this effect on the vasculature could be useful in the treatment of depressed organ function following trauma and hemorrhagic shock. The aim of our study, therefore, was to determine the effect of flutamide on the thoracic aorta and the small vessels of the small intestine in rats.

The results of the present study indicate that flutamide induced significant relaxation of large vessels and small vessels in rats. The effective dose ranges for flutamide-induced relaxation in large and small vessels in male rats were $10^{-5}$M to $16 \times 10^{-5}$M and $0.5 \times 10^{-5}$M to $8 \times 10^{-5}$M, respectively. Our studies have shown that a dose of 25 mg/kg of flutamide significantly improved the function of various organs following trauma and hemorrhage shock in vivo.\(^3\)\(^,\)\(^5\) In a study investigating the pharmacogenetics of flutamide, Radwanski et al\(^18\) found that after oral intake of 250 mg of flutamide there was a rapid peak in its plasma concentration, and after prolonged treatment with flutamide, a steady state, with plasma levels of 112.7 ng/mL (approximately $0.5 \times 10^{-4}$M), developed.\(^18\) We did not measure the plasma levels of flutamide in our experiments; however, in previous studies,\(^1\)\(^3\)\(^,\)\(^5\) after trauma and hem-
Flutamide-induced vascular relaxation in the male aorta in the presence and absence of endothelium. Cumulative dose responses to various concentrations of flutamide in the aortic rings of male rats. A, Rats with or without endothelium. B, Sham or 1.5 hours after trauma and hemorrhage and resuscitation. Values are expressed as percentages of vascular relaxation from initial vascular tension induced by \(2 \times 10^{-5}\) M norepinephrine bitartrate. Data are presented as means ± SE (n=6-7 per group) and were compared by 1-way analysis of variance and Tukey test. Asterisks indicate \(P<.05\) vs with endothelium.

Figure 4.

Flutamide-induced vascular relaxation in the male aorta in the presence and absence of endothelium. Cumulative dose responses to various concentrations of flutamide in the aortic rings of male rats. A, Rats with or without endothelium. B, Sham or 1.5 hours after trauma and hemorrhage and resuscitation. Values are expressed as percentages of vascular relaxation from initial vascular tension induced by \(2 \times 10^{-5}\) M norepinephrine bitartrate. Data are presented as means ± SE (n=6-7 per group) and were compared by 1-way analysis of variance and Tukey test. Asterisks indicate \(P<.05\) vs with endothelium.

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flutamide-induced vascular relaxation is only partially dependent on endothelium. It has been shown that endothelium-derived nitric oxide, a potent endogenous vasodilator, is identical to endothelium-derived relaxing factor in its pharmacological and chemical properties. However, it remains unknown whether flutamide stimulates the release of nitric oxide from vascular endothelial cells to induce vascular relaxation in the intact vessel. Further experiments in this study indicated that flutamide-induced aortic relaxations were not significantly different between sham animals and those subjected to trauma and hemorrhage. Our previous studies have shown that endothelial dysfunction occurs in the very early stage after hemorrhage. In view of this, nitric oxide release does not seem to be responsible for endothelial dependence of flutamide-induced vascular dilation.

It should be noted that flutamide-induced vascular relaxation in the aorta and small intestine was only partially blocked by prior testosterone incubation or endothelial cell removal. Moreover, the flutamide-induced vascular relaxation was only 10% to 20% lower in females than in males. Furthermore, the similar response to flutamide and high doses of testosterone in our experiments indicates that the mechanisms responsible for flutamide-induced vascular relaxation extend beyond testosterone antagonism and may involve nongenomic and endothelial-independent mechanisms. Increased K⁺ conductance, leading to hyperpolarization of vascular smooth muscle, has been proposed as the mechanism underlying the acute inhibition of vascular contraction by estrogen and testosterone. Hyperpolarization of vascular smooth muscle decreases voltage-operated Ca²⁺ channel function, inhibits intracellular Ca²⁺ release, and lowers the ability of Ca²⁺ to activate contractile proteins. It could be speculated that the nongenomic and endothelial-independent mechanisms of flutamide-induced vascular relaxation involves voltage-operated Ca²⁺ channels in vascular smooth muscles, but further studies are needed to determine the exact cellular mechanisms of the direct vascular action of flutamide.

In summary, our results indicate that pharmacological doses of flutamide directly relaxed aortic vessel rings and reduced the perfusion pressure in isolated intestine. This effect was stronger in rings isolated from male rats compared with female rats, but they were not affected by trauma and hemorrhage, and only slightly diminished in vessel rings stripped from the endothelium. Although the inhibition of this action by preincubation of the vessel rings with testosterone suggests an interaction between flutamide and testosterone, and eventually an androgen receptor–dependent mechanism, experiments comparing the effects of flutamide and testosterone in our setup show that the vasorelaxing effect is similar in both agents. Thus, the rapid, nongenomic and endothelium-independent vasorelaxation induced by flutamide in high pharmacological doses appears to be an agonistic effect to testosterone. Further studies should determine the cellular mechanisms of the vasorelaxation after administration of flutamide and its potential therapeutic implications in the treatment of cardiovascular dysfunction after trauma and hemorrhagic shock.

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REFERENCES


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