Testosterone downregulates angiotensin II type-2 receptor via androgen receptor-mediated ERK1/2 MAP kinase pathway in rat aorta

Jay S Mishra, Gary D Hankins and Sathish Kumar

Abstract

Introduction: Blood pressure is lower in females than males. Angiotensin II type-2 receptor (AT2R) induces vasodilation. This study determined whether sex differences in vascular AT2R expression occur and if androgens exert control on AT2R expression in the vasculature.

Methods: AT2Rs in the aorta of male and female Sprague-Dawley rats were examined following alteration in androgen levels by gonadectomy or hormone supplementation.

Results: AT2R mRNA and protein expression levels were lower in the aortas of males than females. In males, testosterone withdrawal by castration significantly elevated AT2R mRNA and protein levels and testosterone replacement restored them. In females, increasing androgen levels decreased AT2R mRNA and protein expression and this was attenuated by androgen receptor blocker flutamide. Ex vivo, dihydrotestosterone downregulated AT2R in endothelium-intact but not endothelium-denuded aorta. Dihydrotestosterone-induced AT2R downregulation in isolated aorta was blocked by an androgen receptor antagonist. Furthermore, blockade of ERK1/2 but not p38 MAP kinase or TGFβ signaling with specific inhibitors abolished dihydrotestosterone-induced AT2R downregulation.

Conclusion: Androgens downregulate AT2R expression levels in aorta, in vivo and ex vivo. The androgen receptor-mediated ERK1/2 MAP kinase-signaling pathway may be a key mechanism by which testosterone downregulates AT2R expression, implicating androgens’ contributing role to gender differences in vascular AT2R expression.

Keywords

Endothelium, ERK, gender difference, blood pressure, testosterone, vascular

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Introduction

The sex difference in blood pressure (BP) has long been recognized between premenopausal women and age-matched men. Before menopause, women have lower BP and are protected from most cardiovascular events compared with age-matched men, and postmenopausal women are at increased risk of cardiovascular complications compared with premenopausal women. The pathophysiological mechanisms have been extensively explored, and increasing evidence has shown that sex hormones are one of the major contributors to the above phenomena. Among different mechanisms, the interaction between sex hormones and the renin-angiotensin system (RAS) is shown to play important role in regulating cardiovascular function and BP. Angiotensin II is the main effector of RAS, and it regulates BP through its effect on the angiotensin II type-1 receptor (AT1R) and angiotensin II type-2 receptor (AT2R). The AT1R promotes antinatriuresis, proliferation, inflammation, and vasoconstriction. AT2Rs are generally assumed to oppose AT1R-mediated responses, for example, by evoking vasorelaxation, natriuresis, antigrowth, and...
anti-inflammatory effects. Increasing evidence has shown that the AT2R is a key player in lowering BP in females but not in males. This enhanced BP-lowering effect of the AT2R in females is attributed to increased expression of the AT2R in females compared to age-matched males. For example, higher AT2R levels are observed in the female brain, kidney, and liver compared to males. However, it is not known if there are differences in expression pattern of AT2Rs in the vasculature between the males and females. The vascular AT2Rs are key elements in homeostatic regulation of the cardiovascular system. Therefore, elucidating the expression patterns of the AT2R in the vasculature would provide evidence for understanding the possible roles of AT2Rs in regulating BP.

Sex steroid hormones, particularly estrogens, are attributed to the greater BP lowering effect and enhanced tissue expression of AT2R in females. However, estrogen is shown to exert a tissue-specific effect by regulating AT2R expression in the kidneys but not in lungs, urethra, and blood vessels. On the other hand, whether androgens exert control on AT2R expression is unknown. Studies show that androgens can regulate RAS components. Therefore, we hypothesized that testosterone (T) is involved in the control of AT2R expression in the vasculature. We investigated (a) whether there are sex differences in vascular AT2R expression, (b) if AT2R is influenced by alterations in androgen status in male and female rats, and (c) the underlying mechanism of AT2R regulation by androgens.

Materials and methods

Animals and institutional animal care and use committee (IACUC) approval

All experimental procedures were carried out in accordance with the National Institutes of Health guidelines (NIH publication no. 85–23 (revised 1996)) with approval by the Animal Care and Use Committee at The University of Texas Medical Branch at Galveston. Three-month-old male and female Sprague-Dawley rats were purchased from Harlan Laboratories, Inc. (Houston, Texas, USA). Rats were housed in a temperature-controlled room (23°C), with a 12L:12D cycle, and with food and water available ad libitum. After one week acclimatization, male rats were divided into three groups: (a) intact, (b) castrated, and (c) castrated with testosterone replacement using subcutaneous implanted pellets (25 mg, 21 day release). Females were divided into four groups: (a) control, (b) treated with dihydrotestosterone (DHT) using pellets (2.5 mg, 21 day release), (c) DHT plus flutamide (100 mg, 21 day release), and (d) flutamide alone. DHT was used in females to overcome its aromatization to estradiol. Castration was done by standard procedures as described in our previous studies. The doses of the pellets were chosen to mimic physiologic hormone levels as reported previously and were further confirmed by hormone assays. After the 21-day treatment, BP was measured and then animals were euthanized by CO2 inhalation, and blood was collected for hormone assays. The thoracic aorta was isolated and either immediately frozen in liquid nitrogen for mRNA and protein analysis or used for ex vivo-signaling studies.

BP measurement

BP was measured using a computerized CODA system (Kent Scientific, Litchfield, Connecticut, USA) as in our previous studies. Briefly, rats were acclimatized for a week to the measurement procedures prior to testing. Rats were held in a preheated restrainer with the tail exposed, and both an occlusion cuff and a volume pressure-recording cuff were placed close to the base of the tail. The cuff was then inflated and deflated automatically within 90 s. BP is measured during 30 consecutive, computer-automated inflation/deflation cycles of the balloon cuff (10 preliminary measurements and 20 test measurements). Unlike other tail-cuff systems, CODA uses volume pressure recording to measure both systolic and diastolic BP, which is then used by the software to calculate the mean BP. Data from the preliminary measurements are discarded and data from the test measurements are averaged. Signals were recorded and analyzed using Kent Scientific software. To minimize stress-induced variations in BP, all measurements were taken by the same person in the same peaceful environment and at the same time of the day.

Hormone assays

T and DHT were measured using enzyme-linked immunosorbent assay (ELISA) kits (T- Enzo Life Sciences, Farmingdale, New York, USA and DHT- BioVendor, Asheville, North Carolina, USA), as in our previous publications. The minimum detectable concentration of testosterone is 6 pg/ml and the intra- and interassay coefficients of variation for testosterone assay was lower than 5%. The minimum detectable concentration of DHT is 6 pg/ml and the intra- and inter-assay coefficients of variation for DHT assay were lower than 8%.

Protein extraction and western blotting

Aorta was homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, Massachusetts, USA) containing a protease inhibitor tablet and phosphatase inhibitor cocktail-2 and -3 (Sigma-Aldrich, St Louis, Missouri, USA). Tissue lysates were centrifuged (14000× g for 10 min at 4°C), and the protein content was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Scientific, Grand Island, New York, USA). The supernatant was resuspended in neutral pH polyacrylamide gel electrophoresis (NuPAGE) lithium dodecyl sulfate sample buffer and reducing agent (Invitrogen; Thermo Scientific). Proteins
(30 µg) alongside Precision Plus Standard (Kaleidoscope; Bio-Rad, Hercules, California, USA) were resolved on 4–12% gradient NuPAGE Bis-Tris gels (Invitrogen) at 100 V for 2 h at room temperature and then transferred onto Immobilon-P membranes (Millipore, Billerica, Massachusetts, USA) at 100 V for 1 h. The membranes were blocked with 5% non-fat dry milk for 1 h and then incubated overnight at 4°C with primary antibodies. The primary antibodies were rabbit monoclonal AT2R (1:3000 dilution; Abcam, Cambridge, Massachusetts, USA) and β-actin (1:5000 dilution; Cell Signaling Technology). After being washed, the membranes were incubated with secondary antibodies (anti-rabbit or -mouse conjugated with horseradish peroxidase) at 1:10000 dilutions and detected with the enhanced chemiluminescence (ECL) detection kits (Pierce; Thermo Scientific). Densitometric measurement was done using ImageJ software.30 Results were expressed as ratios of the intensity of a specific band to that of β-actin.

**Quantitative real-time polymerase chain reaction (PCR)**

Total RNA was extracted using RNeasy mini kit (QIAGEN, Valencia, California, USA) according to manufacturer’s instructions. RNA concentration and integrity was determined using DS-11 spectrophotometer (DeNovix, Wilmington, Delaware, USA). One microgram of total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). After dilution, cDNA corresponding to 100 ng of RNA was amplified by quantitative real-time (qRT)-PCR using FAM (Invitrogen) as the fluorophore in a CFX96 real-time thermal cycler (Bio-Rad). PCR conditions for TaqMan Gene Expression Assay were 2 min at 50°C and 10 min at 95°C for one cycle, then 15 s at 95°C and 1 min at 60°C for 50 cycles. Results were calculated using the 2-ΔΔCT method and expressed in fold change of the gene of interest in treated versus control samples. All reactions were performed in duplicate, and β-actin was used as an internal control. TaqMan assays were carried out in 10-µl volumes for real-time PCR at a final concentration of 250 nM TaqMan probe and 900 nM of each primer. AT2R (Rn00560677_s1) and β-actin (Rn00667869_m1) assays were obtained by Assay-on-Demand (Applied Biosystems; Thermo Scientific).

**Ex vivo treatment to aorta**

Aortas from female rats were dissected, taking care to avoid stretching or compression of the tissues, and placed into ice-cold phosphate-buffered saline (PBS), cleaned of adventitia, and cut into 3–4 rings of approximately 5 mm in length. The rings were placed into 2 ml of Dulbecco’s modified eagle’s medium (DMEM) (Gibco Laboratories; Thermo Scientific) supplemented with 100 µg/ml streptomycin and 100 U/ml penicillin, 1% fetal calf serum (FCS),31 and incubated at 37°C in a humidified 5% CO2 incubator. In some experiments, the endothelium was denuded by gently rubbing the lumen with human hair. The rings were stimulated with DHT at doses of 0, 0.1, 1, and 10 nmol/l for 24 h to examine the dose response of AT2R expression. To inhibit binding of DHT to its receptor, hydroxyflutamide (1 µmol/l) was used. To inhibit DHT-induced extracellular signal-regulated kinases (ERK)1/2 mitogen activated protein (MAP) kinase, p38 MAP kinase, or transforming growth factor (TGF)β activities, inhibitors to ERK1/2 (PD98059, 10 µmol/l and U0126, 10 µmol/l), p38 (SB203580, 10 µmol/l), and TGFβ (SB431542, 10 µmol/l) were used, respectively. Each experiment was repeated at least thrice throughout the study. All chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise noted.

**Statistical analysis**

All data are expressed as the mean±standard error of the mean (SEM). Statistical significance was determined with one-way analysis of variance followed by Bonferroni’s post-hoc test. Comparisons between the two groups were performed using Student t tests. Differences were considered statistically significant at a value of p<0.05. Statistical analysis was conducted using GraphPad Prism (GraphPad, San Diego, California, USA).

**Results**

**BP and hormone measurements**

BP was significantly decreased in castrated rats (111.10±5.2 mm Hg; n=6; p<0.05) compared to intact controls (126.5±2.5 mm Hg; n=6) and testosterone supplementation restored BP to testis-intact controls (129.1±4.1 mm Hg; n=6). In the female rats DHT supplementation increased BP significantly (131.7±5.2; mm Hg; n=5; p<0.05) compared to controls (105.1±2.7; mm Hg; n=6). Plasma testosterone levels were significantly decreased by castration (0.2±0.02 vs 1.4±0.07 ng/ml in intact; n=6 in each; p<0.05) and reinstated to intact levels by replacement (1.5±0.17 ng/ml). In the females, DHT levels were higher in the DHT (186±37.6 pg/ml) and DHT plus flutamide-treated group (179±25.3 pg/ml) compared to controls (111±11.6 pg/ml; n=6 in each; p<0.05). Flutamide alone to females did not alter DHT levels (107±10.4 pg/ml; n=6) compared to vehicle controls.

**AT2R expression is lower in males than females**

To determine whether AT2R expression in the aorta varied between the males and females, mRNA and protein levels of AT2R were determined with quantitative RT-PCR and Western blot analyses. Males had significantly lower AT2R mRNA (↓40%; Figure 1(a)) and protein (↓38%;
AT₂R expression negatively relates to androgen levels in males and females

We next determined whether AT₂R expression in the aorta correlated with an alteration in testosterone levels in males and females. In males, castration significantly elevated AT₂R mRNA (↑52%) and protein (↑76%) expression (Figure 2(a), p<0.05, n=6) compared to intact controls. Testosterone replacement in castrated males restored AT₂R mRNA and protein to levels comparable to that in intact males (Figure 2(a), p<0.05, n=6).

Increasing androgen levels by DHT administration to females significantly decreased AT₂R mRNA (↓53%) and protein (↓27%) expression (Figure 2(b), p<0.05, n=6). Administration of flutamide, an androgen receptor blocker, significantly attenuated the decreased AT₂R mRNA and protein in DHT-treated females (Figure 2(b), p<0.05, n=6). Flutamide by itself did not have any significant effect on AT₂R expression (Figure 2(b), n=6). Thus, testosterone appears to downregulate AT₂R expression in both males and females.

**DHT downregulates AT₂R transcription ex vivo**

Since downregulation of AT₂R by testosterone was apparent, isolated aortas from female rats were used to study the mechanisms by which AT₂R expression is regulated in response to DHT. As shown in Figure 3(a), DHT induced a dose-dependent downregulation of AT₂R mRNA (p<0.05, n=6). Thus, testosterone directly downregulates AT₂R at a transcriptional level.

**DHT downregulates AT₂R in the endothelium but not in vascular smooth muscle**

Studies show that AT₂R is expressed in both the endothelium and vascular smooth muscle layer. To dissect whether the effect of DHT on AT₂R expression occurs in the endothelium or vascular smooth muscle, we used endothelium-intact and endothelium-denuded aorta from female rats. As shown in Figure 3(b), DHT did not alter AT₂R expression in endothelium-denuded aorta but downregulated AT₂R mRNA in endothelium-intact aorta (p<0.05, n=3).

**DHT downregulates AT₂R transcription via androgen receptor-mediated ERK1/2-dependent mechanisms**

We further tested whether activation of the androgen receptor and the downstream signaling of ERK1/2, p38 MAP kinases, and TGF-β are responsible for downregulation of AT₂R expression in response to DHT. Endothelium-intact aortas from female rats were stimulated with DHT in the presence or absence of the androgen receptor antagonist and inhibitors to ERK1/2, p38, or TGF-β. As shown in Figure 4, addition of hydroxyflutamide prevented the reduction of AT₂R expression in response to DHT (p<0.05, n=3). ERK1/2 inhibitor, but not p38 and TGF-β inhibitors, prevented a DHT-induced decrease in AT₂R expression (Figure 5, p<0.05, n=4). Interestingly, p38 MAP kinase and TGF-β inhibitors by themselves decreased basal expression of AT₂R, and DHT in presence of p38 MAP...
Figure 2. Angiotensin II type-2 receptor (AT2R) expression in the aorta relates to androgen levels in male and female rats. AT2R mRNA (upper panel) and protein (lower panel) expression were assessed in aortas isolated from (a) male rats with testes intact, castrated, and castrated with testosterone replacement and (b) female rats treated with vehicle, dihydrotestosterone (DHT), DHT plus flutamide (antiandrogen), and flutamide alone. AT2R mRNA expression was measured by quantitative real-time polymerase chain reaction normalized relative to β-actin levels. AT2R protein expression was determined by Western blotting. Representative Western blots for AT2R and β-actin are shown at the top; blot density obtained from densitometric scanning of AT2R normalized to β-actin is shown at the bottom. Values are given as means±standard error of the mean (SEM) of six rats in each group. *p<0.05 vs vehicle and DHT plus flutamide group. Cas: castration.

Figure 3. Dihydrotestosterone (DHT) downregulates endothelial angiotensin II type-2 receptor (AT2R) expression in isolated aorta. Endothelium-intact and -denuded aorta from female rats were treated with DHT for 24 h, and then AT2R mRNA was measured using quantitative real-time polymerase chain reaction. (a) DHT dose-dependently downregulated AT2R transcription in endothelium-intact aorta. (b) DHT (10 nmol/l) downregulated AT2R transcription only in endothelium-intact but not endothelium-denuded aorta. Values were normalized relative to β-actin levels. Data represent the mean of four independent experiments. *p<0.05 vs vehicle control.
expression relates to androgen status with orchiectomy increasing and testosterone replacement restoring AT2R expression in males, but in the females, increasing DHT levels decreased AT2R expression, which was prevented by blockade of the androgen receptor; (c) DHT directly decreases AT2R expression at transcriptional level in the aorta by decreasing endothelial but not vascular smooth muscle AT2R levels; and (d) DHT downregulates AT2R via androgen receptor-mediated ERK1/2 MAP kinase-dependent mechanisms.

Sex is linked to differences in cardiovascular morbidity and mortality.32,33 The RAS is an important regulatory system that is involved in the long-term control of BP. Angiotensin II is the main effector, which mediates its effect through AT1R and AT2R, which are expressed in cardiovascular system and play an opposite role in BP regulation. AT1R promotes vasoconstriction,34,35 while AT2R promotes vasodilation.36,37 In the present study, we observed that AT2R expression is higher in females compared to males, consistent with the reports in the brain, kidney, and liver.12–14 However, in contrast, spontaneously hypertensive rats (SHRs) showed no sex-dependent differences in AT2R expression in the aorta and mesenteric arteries.17 The reason for the apparent discrepancies between the above study and the present study is not entirely clear. SHRs are hypertensive rats induced by genetic modification and thus may involve different AT2R regulatory mechanisms than in normotensive animals.38,39 In line with increased vascular AT2R expression in females, studies show that AT2R-mediated relaxation is greater in women than men.40 Further experimental studies in rats and mice also support this since C21 (a AT2R agonist) induced a greater increase in renal vasodilation in females than males.13,41 In addition, angiotensin II, at low-dose, reduced pressor response in females that was inhibited by the AT2R antagonist.10 These functional reports together with our molecular finding of increased vascular AT2R expression in females suggest that AT2R may have an important role in contributing to gender differences in vascular tone and BP.

Sex hormones are shown to directly interact with the RAS.42–44 Estrogens are shown to downregulate the vasoconstrictive RAS components (i.e. angiotensin-converting-enzyme (ACE and AT1R)) and upregulate the vasodilatory RAS components (i.e. AT2R and ACE2).15,47 On the other hand, testosterone is shown to upregulate the vasoconstrictive AT1R and ACE.22,48 This is the first study that shows that androgens downregulate vasodilatory AT2R, as observed by the finding that both mRNA and protein expressions of AT2R in aorta are significantly upregulated by orchiectomy and restored by testosterone replacement. In addition, increasing androgen levels in females decreased AT2R expression, and antiandrogen treatment completely normalized AT2R to control levels. Interestingly, the changes in AT2R expression is inversely related to the BP changes observed in these male and female rats. These findings indicate that androgens

**Figure 4.** Dihydrotestosterone (DHT)-mediated downregulation of angiotensin II type-2 receptor (AT2R) transcription is blocked by androgen receptor antagonist. Aortic rings from female rats were treated with DHT (10 nmol/l) in the presence or absence of hydroxyflutamide (1 µmol/l) for 24 h and AT2R mRNA expression was analyzed using quantitative real-time polymerase chain reaction. Data represent the mean of three independent experiments. *p<0.05 vs vehicle control.

**Figure 5.** Dihydrotestosterone (DHT) mediates downregulation of angiotensin II type-2 receptor (AT2R) transcription through ERK-mediated pathways. Aortic rings from female rats were treated for 24 h with DHT (10 nmol/l) in the presence or absence of inhibitors to ERK1/2 (U0126, 10 µmol/l and PD98059, 10 µmol/l), p38 (SB203580, 10 µmol/l), and TGFβ (SB431542, 10 µmol/l). AT2R mRNA expression was analyzed using quantitative real-time polymerase chain reaction and normalized to β-actin. Data represent the mean of four independent experiments. *p<0.05 vs vehicle control, #p<0.05 vs DHT in the absence of any inhibitors. Veh: vehicle.
exert a negative modulatory effect on AT$_2$R expression not only in males but also in females, and may play a role in influencing BP. These findings are clinically relevant since evidence indicates that androgen levels are higher in young women with conditions, such as polycystic ovary syndrome (PCOS), women after menopause, and African American women, and the frequency of hypertension is greater in these populations. It remains to be determined if the vascular AT$_2$R expression is altered in these populations.

More importantly, we showed that DHT was able to downregulate AT$_2$R ex vivo in aorta. These findings suggest that DHT directly induces a downregulation of AT$_2$R transcription independent of any endogenous factors. Because blockade of androgen receptors abolished DHT-induced downregulation of AT$_2$R, we suggest that the effects of testosterone are mediated through androgen receptors. In the present study, a DHT-induced decrease in AT$_2$R transcription is observed in endothelium-intact vessels. No significant difference in DHT-induced AT$_2$R transcription was observed in endothelium-denuded vessels. These findings indicate that testosterone induces reduction in AT$_2$R transcription, primarily in the endothelium rather than in vascular smooth muscle. Although AT$_2$R is expressed in both the vascular smooth muscle and endothelium, the reason why androgens specifically decrease endothelial AT$_2$R is unclear at this time. It would have been ideal to examine if testosterone directly regulates AT$_2$R expression in cultured endothelial cells but this is problematic because endothelial cells rapidly lose AT$_2$R expression when put in culture, thus preventing a study of AT$_2$R expression in cultured endothelial cells.

We next examined the mechanisms by which androgens can downregulate AT$_2$R transcription. Androgens are known to activate p38 and ERK1/2 MAP kinase and TGF-β pathways in the vasculature. The finding that p38 MAP kinase and TGF-β inhibitors by themselves reduced AT$_2$R transcription suggests that basal p38 and TGF-β activities may be important to maintain AT$_2$R expression in unstimulated cells. The inability of p38 MAP kinase and TGF-β inhibitors to prevent a DHT-induced decrease in AT$_2$R transcription suggests the presence of other intracellular mechanisms. Our observation that reduced AT$_2$R expression in response to testosterone was abolished by blocking ERK1/2 suggests that androgen-induced downregulation of AT$_2$R transcription is mediated via the ERK1/2 MAP kinase pathways. Further studies that examine the mechanism by which ERK1/2 MAP kinase downregulates AT$_2$R transcription are warranted. Although this study used aorta, which not only functions as a channel delivering blood to the tissues but also as an important modulator of the entire cardiovascular system by buffering the intermittent pulsatile output from the heart, further studies are necessary to examine AT$_2$R expression and regulation in resistance vessels which play an important role in BP control.

**Conclusions**

Sex differences in vascular AT$_2$R expression is observed with lower levels in males than females. Testosterone downregulates AT$_2$R expression levels in aorta, in vivo and ex vivo. The androgen receptor-mediated ERK1/2 MAP kinase-signaling pathway may be a key mechanism by which testosterone downregulates AT$_2$R expression, implicating androgens’ contributing role to gender differences in vascular AT$_2$R expression.

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