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Prenatal Testosterone Exposure Decreases Aldosterone Production but Maintains Normal Plasma Volume and Increases Blood Pressure in Adult Female Rats[1]

Amar S. More, Jay S. Mishra, Gary D. Hankins, and Sathish Kumar[2]

Division of Reproductive Endocrinology, Department of Obstetrics & Gynecology, The University of Texas Medical Branch at Galveston, Galveston, Texas

ABSTRACT

Plasma testosterone levels are elevated in pregnant women with preeclampsia and polycystic ovaries; their offspring are at increased risk for hypertension during adult life. We tested the hypothesis that prenatal testosterone exposure induces dysregulation of the renin-angiotensin-aldosterone system, which is known to play an important role in water and electrolyte balance and blood pressure regulation. Female rats (6 mo old) prenatally exposed to testosterone were examined for adrenal expression of steroidogenic genes, telemetric blood pressure, blood volume and Na^+ and K^+ levels, plasma aldosterone, angiotensin II and vasopressin levels, and vascular responses to angiotensin II and arg^8-vasopressin. The levels of *Cyp11b2* (aldosterone synthase), but not the other adrenal steroidogenic genes, were decreased in testosterone females. Accordingly, plasma aldosterone levels were lower in testosterone females. Plasma volume and serum and urine Na^+ and K^+ levels were not significantly different between control and testosterone females; however, prenatal testosterone exposure significantly increased plasma vasopressin and angiotensin II levels and arterial pressure in adult females. In testosterone females, mesenteric artery contractile responses to angiotensin II were significantly greater, while contractile responses to vasopressin were unaffected. Angiotensin II type-1 receptor expression was increased, while angiotensin II type-2 receptor was decreased in testosterone arteries. These results suggest that prenatal testosterone exposure downregulates adrenal *Cyp11b2* expression, leading to decreased plasma aldosterone levels. Elevated angiotensin II and vasopressin levels along with enhanced vascular responsiveness to angiotensin II may serve as an underlying mechanism to maintain plasma volume and Na^+ and K^+ levels and mediate hypertension in adult testosterone females.

AGTR1, aldosterone, angiotensin II, blood pressure, plasma volume, pregnancy, testosterone, vascular function, vasopressin

INTRODUCTION

A poor fetal environment and its associated restriction of fetal growth have been consistently linked to adverse phenotypic outcomes in adult offspring, such as increased risk of elevated blood pressure and insulin resistance [1–3]. Nutritional perturbations, especially maternal undernutrition or low protein intake, are recognized as key determinants of a poor fetal environment and are shown to program the adult phenotype. Nevertheless, the relevance of undernutrition and low-protein diet to populations in industrial countries is limited. Recent attention has focused on maternal androgen exposure, because the number of pregnant women with elevated circulating testosterone levels, and their problems with low birth weight and adverse adult health consequences, is rapidly increasing. Higher testosterone levels are reported in several obstetric pathological conditions, such as preeclampsia [4–6], maternal polycystic ovary syndrome (PCOS) [7, 8], obesity [9, 10], stress [11, 12], and smoking [13–15]. In addition, pregnant African American mothers have higher serum testosterone levels [16–18]. Testosterone is an important regulator of growth and differentiation during fetal development [19, 20], and examining the effects of elevated maternal androgen levels are of clinical relevance, since epidemiological evidence shows that elevated maternal testosterone levels are associated with intrauterine growth restriction [21]. Consistently, experimental androgen level increases in pregnant animals are shown to cause intrauterine growth restriction and low birth weight [22–27]. In addition to its effect on fetal growth, elevated testosterone levels during pregnancy have lasting effects in their female offspring, causing reproductive and endocrine disturbances in adult life [28–30]. In fact, elevated androgen levels in pregnant animals are shown to cause hyperactivity of the offspring's hypothalamic-pituitary-gonadal axis and changes in expression of steroidogenic genes in the gonads, leading to increased testosterone production in the adult female [30–33]. In female offspring, prenatal testosterone exposure has been reported to increase blood pressure during adult life [26, 30, 34]. Despite clear evidence that elevated maternal androgens program the increased arterial pressure during adult life, the underlying mechanisms are not completely understood.

Human studies linking low birth weight to an adverse phenotype in adult offspring suggest that the renin-angiotensin-aldosterone system, which has an important effect in regulating blood volume and systemic vascular resistance, contributes to several features of the programmed cardiometabolic phenotype (i.e., hypertension, insulin resistance, hyperleptinemia, etc.) [35]. However, no studies have examined the effect of prenatal testosterone exposure on adrenal development and angiotensin II function. Studies show that the androgen receptor is expressed in fetal adrenals [36], suggesting that androgens may have a direct effect on fetal adrenal development and function. Accordingly, treatment of pregnant rats with

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[2]Correspondence: Sathish Kumar, Department of Obstetrics & Gynecology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1062. E-mail: kusathis@utmb.edu

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letrozole, an aromatase inhibitor, is shown to adversely affect fetal adrenal zone development [37]. In vitro studies show that androgens decrease the activity of many steroidogenic enzymes (e.g., 3β-hydroxysteroid dehydrogenase [3β-HSD] [38], 21-hydroxylase [39], and 11β-hydroxylase [40]). Testosterone also exerts a direct inhibitory effect on basal aldosterone release [41]. In addition, studies indicate that testosterone interacts directly with the renin-angiotensin system, activating the classical constrictor pathway via upregulation of angiotensin II/angiotensin type-1 receptor (AGTR1) pathways [42–46]. Therefore, in the present study, we investigated whether prenatal testosterone exposure alters adrenal expression of key genes involved in steroidogenesis and their hormonal production. Since changes in adrenal hormones directly regulate fluid balance and arterial pressure, we measured plasma volume, serum and urinary Na^+ and K^+ levels, and systemic blood pressure. In addition, we measured plasma angiotensin II levels and their effects on vascular contraction. Herein, we present evidence in an in vivo pregnancy rat model system that prenatal testosterone exposure causes a selective decrease in adrenal *Cyp11b2* mRNA transcripts, leading to a decrease in plasma aldosterone levels. However, normal plasma volume and Na^+ and K^+ balance are maintained along with a paradoxical increase in systemic blood pressure in prenatal testosterone-exposed rats. Elevated plasma angiotensin II and arg^8-vasopressin (AVP) levels, along with enhanced vascular responsiveness to angiotensin II, may contribute to maintenance of plasma volume and Na^+ balance and mediate hypertension in prenatal testosterone-exposed adult rats.

MATERIALS AND METHODS

Testosterone Treatment and Animal Care

The experimental protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas Medical Branch at Galveston (Galveston, TX), and procedures were in accordance with the National Institutes of Health (NIH) guidelines (NIH Publication No. 85–23, revised 1996) for care and use of animals. On Day 12 of pregnancy, Sprague-Dawley rats were purchased from Harlan laboratories and were maintained on 12L/12D cycles in a temperature-controlled room (23°C) and provided with food and water ad libitum. On Day 15 of pregnancy, rats were divided into control and treatment groups. The control group received vehicle (sesame oil; $n = 6$) subcutaneously, and the treatment group received testosterone propionate (0.5 mg/kg; $n = 6$) subcutaneously from Day 15 to 19 of gestation, as previously described [47]. This dose and duration of testosterone propionate were selected to mimic increases in testosterone levels in preeclamptic pregnant women [25, 27, 48, 49]). This treatment paradigm led to an increase in plasma testosterone levels in testosterone-treated dams, 2.2 ± 0.23 ng/ml compared to 1.0 ± 0.25 ng/ml in vehicle-treated control dams ($n = 6$ in each group; $P < 0.05$). Rats were allowed to deliver normally. The number of pups per control and testosterone-treated mother were kept at 10 to ensure equal nutrient access for each pup. The ratio between males and females was kept equal, when possible. On weaning, pups were separated from their mothers; only female pups were used in this study. Pups were fed a regular diet ad libitum. At 24 wk of age, changes in plasma volume and mean arterial pressure were measured. Then, the rats were killed by CO_2 inhalation, plasma/serum was separated for hormone measurements, adrenal glands were collected for steroidogenic and angiotensin II receptors gene expression, and mesenteric arteries were isolated for protein expression and vascular reactivity studies. Animal diestrus stage based on sequential vaginal cytology [50] was used for all studies.

Adrenal Steroidogenic and Angiotensin II Receptor Gene Expression

Total RNA was extracted from snap-frozen adrenal glands using the RNAeasy kit (QIAGEN, Valencia, CA), which included a deoxyribonuclease step, according to the manufacturer's instructions. Total RNA concentration and purity were assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Further cDNA synthesis was done from 1 μg of total RNA by using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA was diluted 1:10, and 2 μl of resulting cDNA was amplified by quantitative real-time PCR using SYBR Green (Bio-Rad) as fluorophore in a CFX96 real-time thermal cycler (Bio-Rad). A specific pair of primers (Integrated DNA Technology, San Jose, CA) were used for each gene amplification: *star* forward, 5′-AGG AAA GCC AGC AGG AGA ATG-3′, reverse, 5′-GTC CAT GGG CTG GTC TAG CA-3′; *Cyp11a1* forward, 5′-TCA AGC AGC AAA ACT CTG GA-3′, reverse, 5′-CGC TCC CCA AAT ACA ACA CT-3′; *Hsd3b1* forward, 5′-AGG GCA TCT CTG TTG TCA TCC AC-3′, reverse, 5′-TGC CTT CTC GGC CAT CCT TT-3′; *Cyp11b1* forward, 5′-GGC ACA TAC GAG CTG GTG AGT-3′, reverse, 5′-GTC CTC CTG CCT GCA TCT CT-3′; *Cyp11b2* forward, 5′-TGC TGC TTG GGC AAA GGT-3′, reverse, 5′-CTT TTC GCC CTA CCG ACT TG-3′; *Agtr1* forward, 5′-AAC AAC TGC CTG AAC CCT CT-3′, reverse, 5′-TCA GAA CAA GAC GCA GGC T-3′; *Agtr2* forward, 5′-TCT GTC TCA AAG AAG GAA TCC CC-3′, reverse, 5′-CCA ACA CAA CAG CAG CTG C-3′; *ubiquitin* forward, 5′-CTTTTGTGAGGACTGCAGCCAACA-3′, reverse, 5′-AGGGTGATGGTCT TACCAGTTAAGGT-3′. PCR conditions used were 95°C for 10 min for 1 cycle and 94°C for 15 sec, 60°C for 30 sec, and 72°C for 15 sec for 45 cycles, followed by a final dissociation step (0.05 sec at 65°C and 0.5 sec at 95°C). Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold changes of expression of genes of interest. All reactions were performed in duplicate.

Aldosterone Measurements

Plasma aldosterone was measured using an aldosterone enzyme immunoassay kit (Enzo Life Sciences, Farmingdale, NY), per the manufacturer's instructions. The intra- and interassay coefficients of variation for the assay were 4.2% and 10.9%, respectively, and sensitivity was 4.7 pg/ml.

Blood and Plasma Volume

Blood and plasma volumes were determined using Evans blue dye, as described previously [51, 52]. Rats were anesthetized with isoflurane and placed on a heated table to maintain body temperature at 37 ± 1°C. The right femoral artery and vein were cannulated with PE-50 tubing, and an arterial blood sample was collected for determination of hematocrit and a ''blank'' for Evans blue measurement. Then, 0.3 ml Evans blue dye (0.5 mg/ml; Sigma, St. Louis, MO) was injected into the venous line. An additional 0.5 ml of isotonic saline was also injected to ensure that all Evans blue was delivered to the rat. The dye was allowed to circulate for 5 min, after which a final blood sample was taken. Blood was collected into EDTA microtubes and centrifuged at 3000 rpm for collection of plasma, and samples were added in duplicate to a 96-well microplate and read at 620 nm (BMG Labtech Gmbh, Ortenberg, Germany). Baseline plasma samples from each animal acted as a blank for that individual animal. A standard curve was constructed with known concentrations of Evans blue. The inter- and intra-assay coefficients of variation for Evans blue were 10.0% and 4.3%, respectively. Plasma volume was then calculated as: mg dye injected/corrected plasma dye concentration. Blood volume was calculated as: plasma volume/$(1 - [0.009 \times \text{haematocrit}])$ using an F-cells ratio of 0.9 to account for the difference between whole-body and venous haematocrit [53].

Na^+ and K^+ Measurements

Serum and urine Na^+ and K^+ levels were measured using a VITROS 5600 Integrated System (Raritan, NJ). A 24-h urine collection was done by placing the animals in individual metabolic cages. Briefly, 10 μl of the sample and reference fluids were deposited on separate halves of the VITROS Clinical Chemistry Slide containing ion-selective electrodes (Na^+ and K^+). A paper bridge connects the reference electrode and the sample electrode. A stable liquid junction between the two fluids is formed in the paper bridge in approximately 20 sec. After the completion of a 3-min incubation period, the electrometer in the VITROS Analyzer measured the potential difference between the reference and indicator electrodes.

Blood Pressure

Blood pressure in conscious, free-moving rats was determined using a telemetry system, as we described previously [48, 54, 55]. Briefly, rats were anesthetized with 2.5% isoflurane, and a flexible catheter attached to a radio transmitter (model TA11PA-C10; Data Sciences, Minneapolis, MN) was inserted into the left femoral artery. After surgery, rats were housed in individual cages and allowed to recover for 1 wk. Blood pressure levels were recorded continuously for 7 days. Blood pressure measurements obtained with a 10-sec sampling period were averaged and recorded every 10 min, 24 h/day, using software (Dataquest version 4.0; Data Sciences) provided by the manufacturer.

Serum Norepinephrine Levels

The competitive noradrenaline ELISA kit (Eagle Biosciences, Nashua, NH) were used to measure the norepinephrine level in plasma samples by manufacturer's instruction. The intra- and interassay coefficients of variation of the assay were less than 10%, respectively, and sensitivity was 16 pg/ml.

Plasma Angiotensin II, AVP, and Insulin Levels

A commercial enzyme immunoassay kit was used to determine plasma levels of angiotensin II (Phoenix Pharmaceutical Inc., Burlingame, CA), AVP (Enzo Life Sciences, Farmingdale, NY), and insulin (Mercodia, Uppsala, Sweden). All procedures were conducted according to the assay kit instructions. The intra- and interassay coefficients of variation for the angiotensin II assay were 5%–10% and <15%, respectively, and sensitivity was 0.09 ng/ml. The intra- and interassay coefficients of variation for the AVP assay were 8.2% and 9.0%, respectively, and sensitivity was 2.84 pg/ml. The intra- and interassay coefficients of variation for insulin assay was 1.8% and 3.3 %, respectively, and sensitivity was 0.07 $g \cdot L^{-1}$.

Vascular Reactivity

Resistance mesenteric arteries (2-mm segments of the third-order branch of the superior mesenteric artery (MA), 150–200-μm diameter) were dissected free of fat and connective tissue and mounted in Mulvany-style isometric wire myographs (Danish Myotechnology, Aarhaus, Denmark) for vessel reactivity assessment. Vessels were maintained at 37°C in physiologic Krebs buffer consisting of 120 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 11.0 mM dextrose, and 1.8 mM $CaCl_2$, aerated with 95% O_2 and 5% CO_2 (pH, 7.4). Before mounting, endothelium was removed by gently rubbing the intimal surface of rings with a tungsten wire. The rings were bathed in 6 ml of Krebs buffer and allowed to equilibrate for 60 min before normalization to an internal diameter of 0.9 of $L_{13.3\ kPa}$ by using normalization software (Myodata; Danish Myotechnology). The rings were then assessed for vascular function. Data were captured using a data acquisition system (Power Lab; AD Instruments, Colorado Springs, CO). The arterial rings were exposed to 80 mM KCl until reproducible depolarization-induced contractions were achieved. After a second round of washing and equilibration with Krebs buffer, endothelium removal was verified by the absence of relaxation to acetylcholine (10 μM) in rings precontracted with phenylephrine (3 μM). Then, vascular contractile responses to cumulative additions of angiotensin II and AVP were determined.

Western Blot Analysis

A protocol similar to those in our previous studies was followed [56]. In brief, mesenteric arteries were homogenized in a 1× radioimmunoprecipitation assay buffer (4°C; Cell Signaling Technology, Danvers, MA) containing a protease inhibitor tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 2 and 3 (Sigma). Lysates were then sonicated with three 5-sec bursts with 30% power and were spun at 14 000 × *g* for 10 min. Total proteins were quantified using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Proteins (30 μg) in supernatant were suspended in NuPAGE lithium dodecyl sulfate sample buffer and reducing agent (Invitrogen, Carlsbad, CA) and resolved on 4%–12% precasted gradient polyacrylamide gels (NuPAGE Life technologies, Carlsbad, CA) alongside Precision Plus Standard (Kaleidoscope; Bio-Rad) at 100 V for 2 h. Furthermore, proteins in gel were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) by electroblotting at 100 V for 1.5 h. Membranes were blocked with 5% nonfat dried milk for 1 h and then incubated with primary antibodies at 4°C overnight. Antibodies for AGTR1 (1:500 dilution), angiotensin II type-2 receptor (AGTR2) (1:500 dilution), and β-actin (1: 5000 dilution) were obtained from BD Transduction Labs (Franklin Lakes, NJ), Abcam (Cambridge, U.K.), and Cell Signaling Technology, respectively. After incubation and washing, membranes were incubated with secondary antibodies (anti-mouse- or anti-rabbit-conjugated with horseradish peroxidase, Southern Biotech, Birmingham, AL) at 1:12 000 dilutions for 1 h and detected with an ECL detection kit (Millipore). After development, a densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD; http:/rsh.info.nih.gov/ij/). Results were expressed as ratios of the intensity of specific band to that of β-actin.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). For cumulative concentration-response curves, analysis was done using computer fitting to a four-parameter sigmoid curve, using Prism 6 software (GraphPad Software Inc., La Jolla, CA) to evaluate the negative log of half-maximal effective concentration (pD2) and maximum asymptote of the curve (E_{max}). Comparisons between the groups were performed using unpaired Student *t*-tests. Data are presented as means ± SEM. Differences were considered significant at $P < 0.05$. The number of litters studied is represented by "*n*."

RESULTS

Adrenal Steroidogenic and Angiotensin II Receptor Gene Expression

To address the effect of prenatal testosterone exposure on adrenal steroidogenesis, we determined the mRNA expression of major steroidogenic components. There were no significant changes in *star*, *Cyp11a1*, *Hsd3b1*, or *Cyp11b1* mRNA levels (Fig. 1, A–D; n = 6 in each group). The mRNA levels of *Cyp11b2*, the gene for aldosterone synthase, which is the rate-limiting enzyme involved in aldosterone biosynthesis, was significantly decreased by 40% in prenatal testosterone-exposed rats compared to controls (Fig. 1E; n = 6; $P < 0.05$).

Since angiotensin II regulates aldosterone synthesis via its receptors, we measured *Agtr1* and *Agtr2* mRNA levels in adrenals. The mRNA expression of *Agtr1* was significantly increased by 60% in the prenatal testosterone-exposed group compared to controls (Fig. 2A; n = 6; $P < 0.05$), whereas mRNA expression of *Agtr2* was significantly decreased by 60% in the prenatal testosterone-exposed group compared to controls (Fig. 2B; n = 6; $P < 0.05$).

Plasma Aldosterone Levels

To determine whether aldosterone levels were altered due to the decreased *Cyp11b2* expression in the adrenals in prenatal testosterone-exposed rats, plasma levels of aldosterone were measured. As shown in Figure 3, plasma levels of aldosterone were significantly reduced in prenatal testosterone-exposed rats (63.17 ± 7.75 pg/ml) compared to controls (171.80 ± 27.71 pg/ml) (n = 6; $P < 0.05$).

Plasma and Blood Volume and Serum and Urinary Na^+ And K^+ Levels

Decreased aldosterone levels in prenatal testosterone rats may have important effects on water and electrolyte balance as well as systemic arterial blood pressure. Therefore, we determined the effects of prenatal testosterone exposure on blood volume and Na^+ and K^+ levels. Plasma and blood volumes did not differ between control and prenatal testosterone-exposed rats (Fig. 4, A and B; n = 6). The serum Na^+ and K^+ levels were comparable between the prenatal testosterone-exposed rats and controls (Fig. 4, C and D; n = 6). Similarly, Na^+ and K^+ levels in the urine did not significantly differ between prenatal testosterone-exposed rats and controls (Fig. 4, E and F; n = 6).

Systemic Blood Pressure

To determine if prenatal testosterone exposure had an effect on blood pressure, intravascular telemetric transmitters were placed and, 7 days later, blood pressure was monitored continuously. Blood pressure was significantly higher in the prenatal testosterone-exposed rats (112 ± 1.8 mm Hg; n = 6) compared to controls (104 ± 1.6 mm Hg; n = 6; $P < 0.05$) (Fig. 5A). Heart rate was also significantly higher by approximately 15 beats/min in prenatal testosterone-exposed

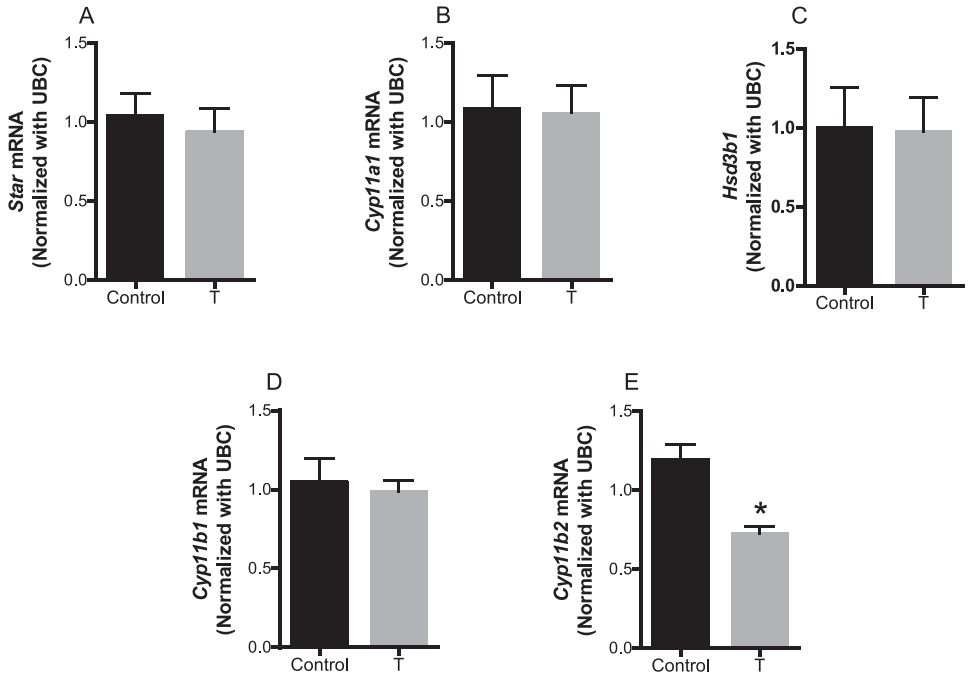

FIG. 1. Effect of prenatal testosterone (T) exposure on mRNA expression of major steroidogenic genes in adrenals of adult female rats. Real-time reverse transcriptase PCR was used to assess adrenal *Star* (**A**), *Cyp11a1* (**B**), *Hsd3b1* (**C**), *Cyp11b1* (**D**), and *Cyp11b2* (**E**) mRNA expression. Quantitation of adrenal steroidogenic genes was normalized relative to ubiquitin (UBC) levels. Values are presented as mean ± SEM of six animals in each group of animals. $*P < 0.05$ vs. control.

rats (380 ± 3.8 beats/min; n = 6) compared to controls (365 ± 6.2 beats/min; n = 6; $P < 0.05$ [Fig. 5B]).

Serum Norepinephrine Levels

We next determined if the increased heart rate in prenatal testosterone-exposed rats relates to altered sympathetic activity. We measured serum norepinephrine levels, which is a proxy for sympathetic activity. As shown in Figure 6, the levels of norepinephrine were not significantly different between the control (n = 6) and prenatal testosterone-exposed rats (n = 6).

Plasma Angiotensin II, AVP, and Insulin Levels

In search of mechanisms that could contribute to maintenance of Na^+ and K^+ levels, as well as increased systemic blood pressure, we examined plasma angiotensin II and AVP levels, which are potent regulators of electrolyte balance and blood pressure. As shown in Figure 7A, plasma angiotensin II levels were significantly higher in prenatal testosterone-exposed rats (1.05 ± 0.07 ng/ml) compared to controls (0.65 ± 0.03 ng/ml; n = 6 in each group; $P < 0.05$). In addition, the plasma AVP levels were significantly higher in prenatal testosterone-exposed rats (28.203 ± 0.7 ng/ml) compared to

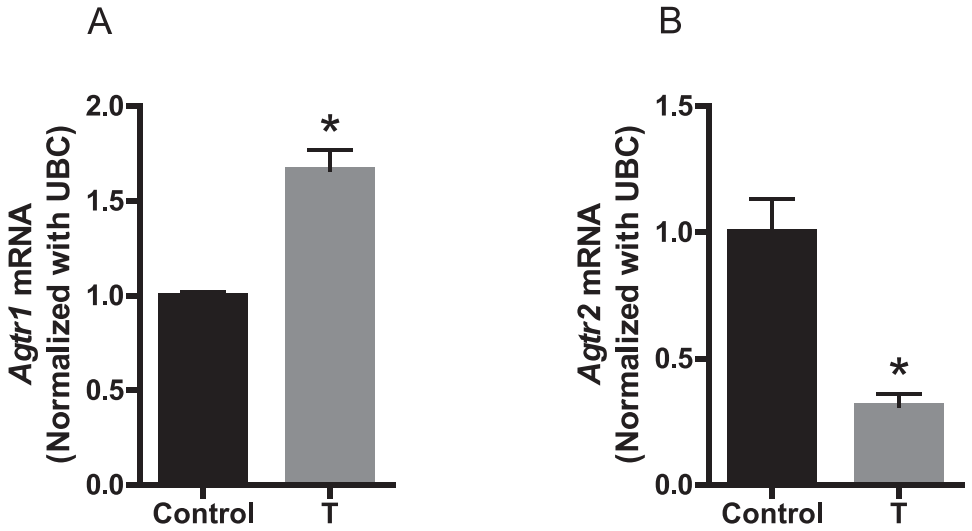

FIG. 2. Effect of prenatal T exposure on angiotensin II receptors expression in adrenals of adult female rats. *Agtr1* (**A**) and *Agtr 2* (**B**) mRNA levels were measured using real-time reverse transcriptase PCR. Gene expression was normalized by using ubiquitin (UBC) levels. Values are presented as mean ± SEM of six animals in each group of animals. $*P < 0.05$ vs. control.

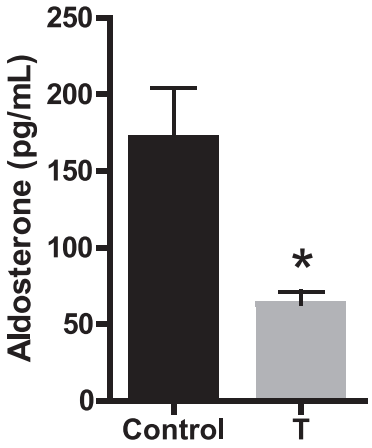

FIG. 3. Effect of prenatal T exposure on plasma aldosterone levels in adult females. Plasma was separated from blood that was collected through cardiac puncture following CO_2 inhalation. Aldosterone levels were determined using enzyme immunoassay. All data are expressed as mean ± SEM of six animals in each group. *$P < 0.05$ vs. control.

controls (21.325 ± 0.7 ng/ml; n = 6 in each group; $P < 0.05$) (Fig. 7B). In addition, insulin, which is also known to play a significant role in increasing renal Na^+ reabsorption, was significantly higher in prenatal testosterone-exposed rats (118.31 ± 36.68 pmol/L; n = 6; $P < 0.05$) compared to controls (43.59 ± 9.58 pmol/L; n = 6) (Fig. 7C).

Vascular Reactivity

Vascular contractile responses to KCl, a determination of depolarization-induced vessel contraction was similar in arterial rings from prenatal testosterone-exposed rats (7.3 ± 0.73 mN) compared to controls (7.5 ± 0.73 mN). In both control and prenatal testosterone-exposed rats, angiotensin II induced a dose-dependent increase in contractile responses in resistance mesenteric arteries, which are actual determinants of systemic blood pressure. However, the angiotensin II-induced contractile responses were exaggerated, with a leftward shift in the dose-response curves in the prenatal testosterone-exposed rats (pD2, 9.08 ± 0.10; n = 6; $P < 0.05$) compared to control rats (pD2, 8.61 ± 0.08; n = 6; $P < 0.05$; Fig. 8A). Similarly, the angiotensin II-induced maximal responses were greater in the prenatal testosterone-exposed rats ($E_{max} = 60.9 \pm 6.93$; n = 6; $P < 0.05$) than controls ($E_{max} = 33.3 \pm 7.95$; n = 6; $P < 0.05$; Fig. 8A). On the other hand, contractile responses to AVP were not altered in prenatal testosterone-exposed rats compared to controls (n = 6 in each group; Fig. 8B).

Mesenteric Arterial Expression of Angiotensin II Receptors

To determine whether angiotensin II receptor expression in the mesenteric arteries correlated with alteration of angiotensin II contractile responses in testosterone rats, protein levels of angiotensin II receptors were determined using Western blot analyses. As shown in Figure 9, prenatal testosterone-exposed rats had a significant increase in AGTR1 in mesenteric arteries compared to controls (n = 6 in each group; $P < 0.05$). In contrast, AGTR2 was significantly decreased in mesenteric arteries of prenatal testosterone-exposed rats (n = 6) compared to controls (n = 6; $P < 0.05$; Fig. 9). Thus, prenatal testosterone-exposed rats have a significantly increased AGTR1/AGTR2 ratio in mesenteric arteries by approximately 1.6 fold compared to controls (n = 6; $P < 0.05$; Fig. 9).

DISCUSSION

For the first time, we show that prenatal testosterone exposure downregulates adrenal *Cyp11b2* mRNA transcripts, leading to a decrease in plasma aldosterone levels. Despite lower aldosterone levels, normal plasma volume and Na^+ and K^+ balance, along with a paradoxical increase in blood pressure, are observed in prenatal testosterone-exposed rats. Elevated plasma angiotensin II, AVP, and insulin levels, along with enhanced mesenteric arterial contractile responsiveness to angiotensin II, may contribute for maintenance of normal plasma volume and Na^+ and K^+ balance and mediate hypertension in prenatal testosterone-exposed adult females.

Several lines of evidence suggest that elevated testosterone during pregnancy causes fetal growth restriction [22–27] and PCOS-like phenotype [28–30], including cardiovascular dysfunction and hypertension, in adult females [26, 30, 34]. Consistent with evidence showing that boys and girls of PCOS mothers are often hyperandrogenic [57, 58], we and others using the model of prenatal androgen exposure have shown that adult females produce higher testosterone levels with associated increases in arterial pressure [26, 30, 34, 54, 55]. Intriguingly, this study shows that hypertensive response in prenatal testosterone-exposed adult females was associated with lower plasma levels of aldosterone. Consistently, the adrenal *Cyp11b2* that encodes the enzyme responsible for aldosterone biosynthesis was downregulated in the prenatally testosterone-exposed animals.

Angiotensin II also regulates aldosterone biosynthesis [59, 60]; in the current investigation, *Agtr1* expression was increased, whereas *Agtr2* was decreased, in prenatal testosterone-exposed adrenals. Previous studies have shown that AGTR1 is predominantly expressed in the zona glomerulosa, and its activation leads to an increase in aldosterone production, while AGTR2 is almost exclusively localized in the medulla, and its activation increases catecholamine production [61]. The finding that aldosterone levels are lower in prenatal testosterone-exposed rats, despite increased angiotensin II levels and enhanced *Agtr1* expression, suggests that the decreased *Cyp11b2* expression may play a critical role in contributing to reduced aldosterone synthesis. In addition, reduced *Agtr2* expression may be a reason for the lack of increase in norepinephrine levels.

Aldosterone is an antinatriuretic factor that is essential for proper Na^+ balance and regulation of plasma volume [62, 63]. Hypoaldosteronism, observed in the current study, should theoretically decrease blood pressure by promoting inappropriate renal Na^+ and water loss [64–66]. The findings that prenatal testosterone-exposed adult rats maintained normal plasma volume and Na^+ and K^+ balance and increased systemic blood pressure, in spite of having low plasma aldosterone levels, indicate a role for other hormones and paracrine factors in modulating Na^+ absorption, plasma volume, and blood pressure.

The next question arises as to what factors contribute to maintenance of normal Na^+ and K^+ balance and higher blood pressure in prenatal testosterone-exposed adult rats. AVP is known to play an important role in restoring Na^+ and K^+ balance, especially in conditions of adrenal insufficiency [67–70]. Thus, the higher AVP levels observed in prenatal testosterone-exposed adult rats may contribute to a normal Na^+ and K^+ balance. Whether the AVP increase in prenatal testosterone-exposed rats is secondary to altered plasma osmolarity, or if testosterone directly upregulates AVP synthesis [71, 72] and function [73], remain to be established. In addition, higher plasma levels of angiotensin II and insulin

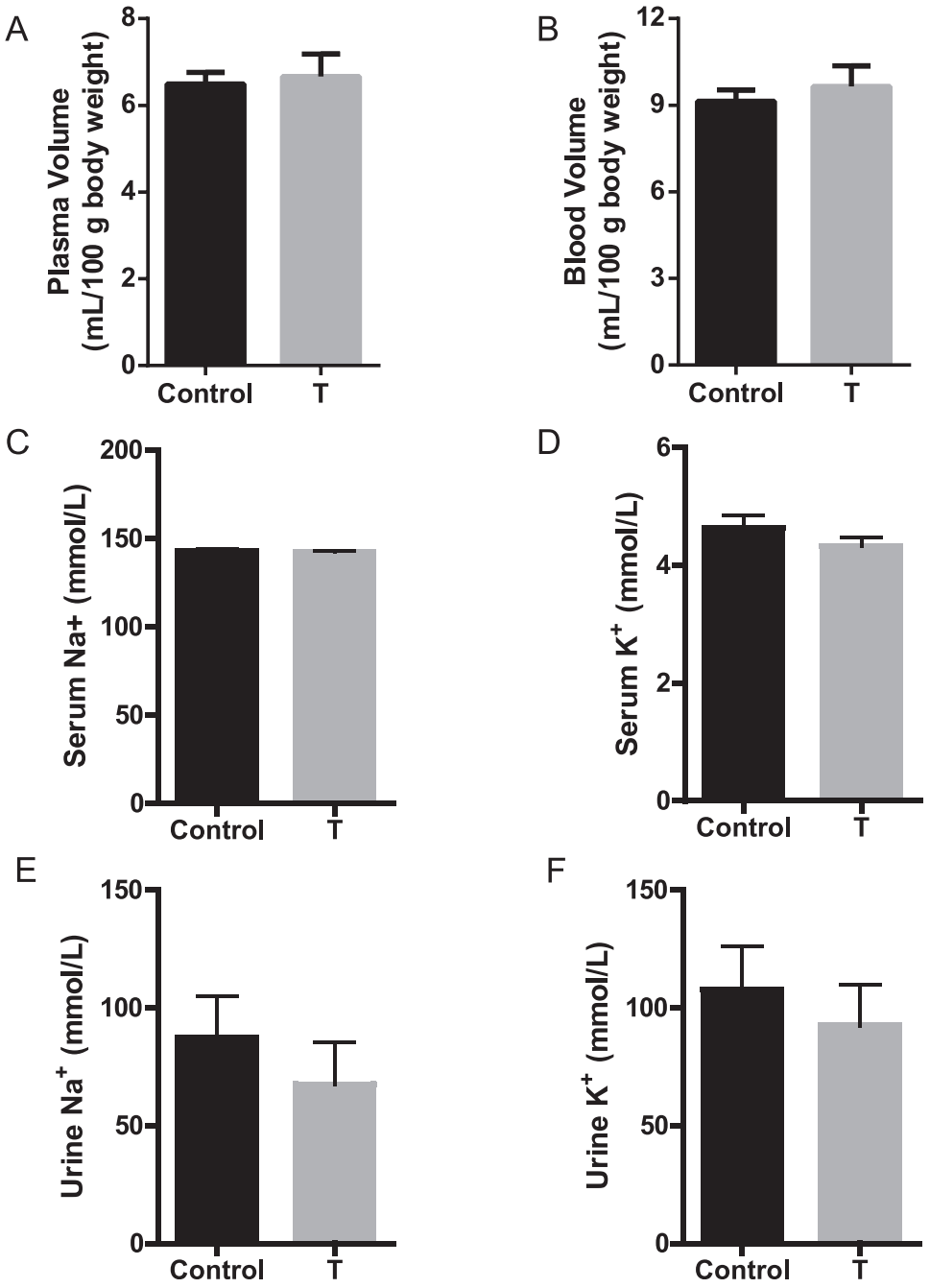

FIG. 4. Effect of prenatal T exposure on plasma and blood volume and serum and urine Na^+ and K^+ levels in adult females. Plasma (**A**) and blood (**B**) volumes were determined using Evans blue dye. Serum Na^+ (**C**) and K^+ (**D**) levels and urine Na^+ (**E**) and K^+ (**F**) levels were measured using VITROs 5600 Integrated System. All data are expressed as mean ± SEM of six animals in each group.

in prenatal testosterone-exposed rats may also contribute to renal salt and water reabsorption [74–77]. Thus, increases in plasma AVP together with increases in angiotensin II and insulin levels in prenatal testosterone-exposed rats may contribute to maintenance of normal plasma volume and electrolyte balance.

In search of the mechanisms that contribute to an increase in systemic blood pressure, we found that the prenatal testosterone-exposed rats have elevated heart rates, which supports the possibility that sympathetic nervous system activation may be involved [78]. A recent study using analysis of heart rate variability to assess cardiac autonomic function [79] showed increased sympathetic and decreased parasympathetic frequency components in young PCOS women. However, the finding of normal plasma norepinephrine levels in these rats indicates that global sympathetic activity is unlikely to be increased. However, further studies of more accurate assessments of sympathetic transmitter release using radioisotope dilution measurements of total body or regional norepinephrine spillovers [80] or direct sympathetic nerve recordings [81] are warranted to definitively discount the role of sympathetic activity. It is possible that altered control of cardiac parasympathetic activity or changes in heart morphology may contribute to increased heart rate [82]. In addition, impaired cardiac function may also be secondary to altered metabolic and hemodynamic functions [83, 84]. Furthermore, increased

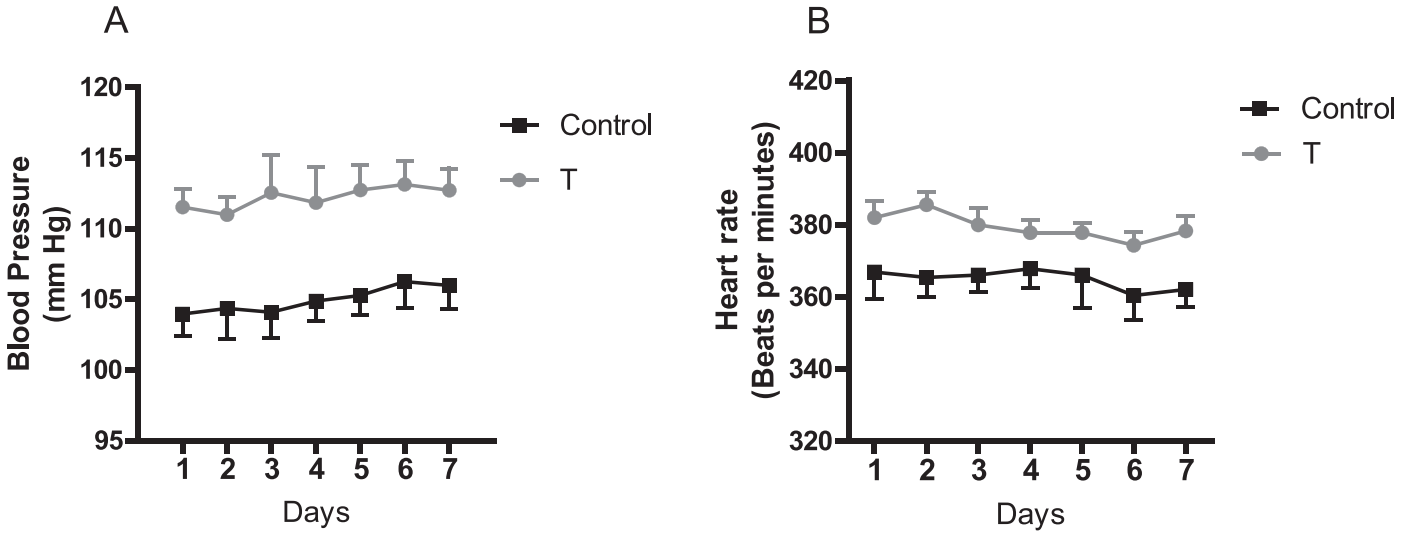

FIG. 5. Effect of prenatal T exposure on systemic blood pressure and heart rate in adult females. Blood pressure (**A**) and heart rate (**B**) were continuously monitored via telemetry catheters in the femoral artery. Blood pressure and heart rate values are presented in 24-h intervals. Data points represent the mean ± SEM of measurements in six rats in each group. *$P \leq 0.05$ vs. control.

angiotensin II and insulin levels in prenatal testosterone-exposed rats may contribute to increasing heart rate [85–87]. Further investigation on an ultrastructural, biochemical, and genetic basis may prove revealing.

Increased heart rate may contribute to increased blood pressure. To determine the contribution of the vasculature reactivity to blood pressure increase, we examined vascular responses to angiotensin II and AVP. In the present study, angiotensin II-induced contractile responses were significantly increased: both the pD2 and E_{max} were greater in prenatal testosterone-exposed rats compared to controls. Because prenatal testosterone exposure caused increases in angiotensin II-induced contractions in the absence of functional endothelium, we suggest that the enhanced arterial sensitivity to angiotensin II primarily occurred in the vascular smooth muscle cells. Angiotensin II acts through AGTR1 and AGTR2. AGTR1 is known to mediate most of the well-known effects of angiotensin II, including vasoconstriction and hypertension [88]. Actions of the AGTR2 are less clear, but they seem to counterbalance some of the actions of the AGTR1 leading to vasodilation. In the present study, we show that the expression of AGTR1 in the mesenteric arteries was increased, while AGTR2 was decreased in prenatal testosterone-exposed rats compared to controls. Studies indicate that the AGTR1/AGTR2 ratio plays a crucial role in the development of the hypertensive phenotype [89], and the vascular AGTR1/AGTR2 ratio relates to the magnitude of blood pressure elevation observed in spontaneously hypertensive rats [89]. Thus, the ratio of AGTR1/AGTR2 was significantly higher in prenatal testosterone-exposed rats. Interestingly, the vasomotor response to other potent constrictors, such as AVP, was not enhanced in prenatal testosterone-exposed rats. Other studies of developmentally programmed hypertension have also found no modification in vascular responses to phenylephrine in mesenteric arteries [26, 54]. Thus, it is likely that the effect of prenatal programming on vasoconstrictors is agonist dependent. In addition, these findings suggest that prenatal testosterone-mediated vascular programming occurs at the agonist-specific level rather than at common intracellular signaling pathways. Thus, higher angiotensin II levels and the associated vascular hyperresponsiveness to angiotensin II may contribute to the increased blood pressure in prenatal testosterone-exposed females. Consistently, treatment with enalapril, which inhibits angiotensin II synthesis, is shown to restore blood pressure in prenatal testosterone-exposed adults [90].

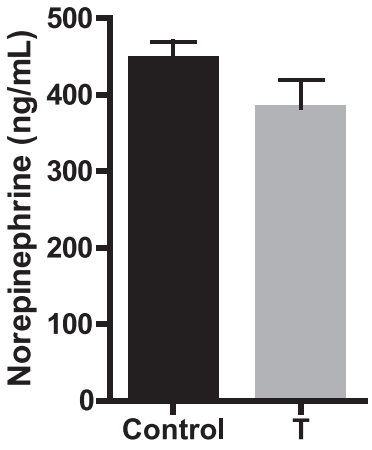

FIG. 6. Effect of prenatal T exposure on serum norepinephrine levels in adult females. Norepinephrine levels were determined using enzyme immunoassay. All data are expressed as mean ± SEM of six animals in each group.

In conclusion, prenatal testosterone exposure exhibits a reduction in *Cyp11b2* expression with an associated decrease in plasma aldosterone levels. However, normal plasma volume and Na^+ and K^+ balance are maintained, possibly because of elevations in AVP, angiotensin II, and insulin. It is unclear whether the latter is a compensatory response or an effect of prenatal testosterone programming. Nevertheless, because high levels of AVP and angiotensin II levels coincide with an increased vascular AGTR1/AGTR2 ratio and exaggerated vascular responsiveness to angiotensin II, they may contribute to hypertension. Thus, it is possible that elevated blood pressure mediated by increased AVP and angiotensin II levels in prenatal testosterone-exposed rats may be a tradeoff for maintaining an optimal water and electrolyte balance.

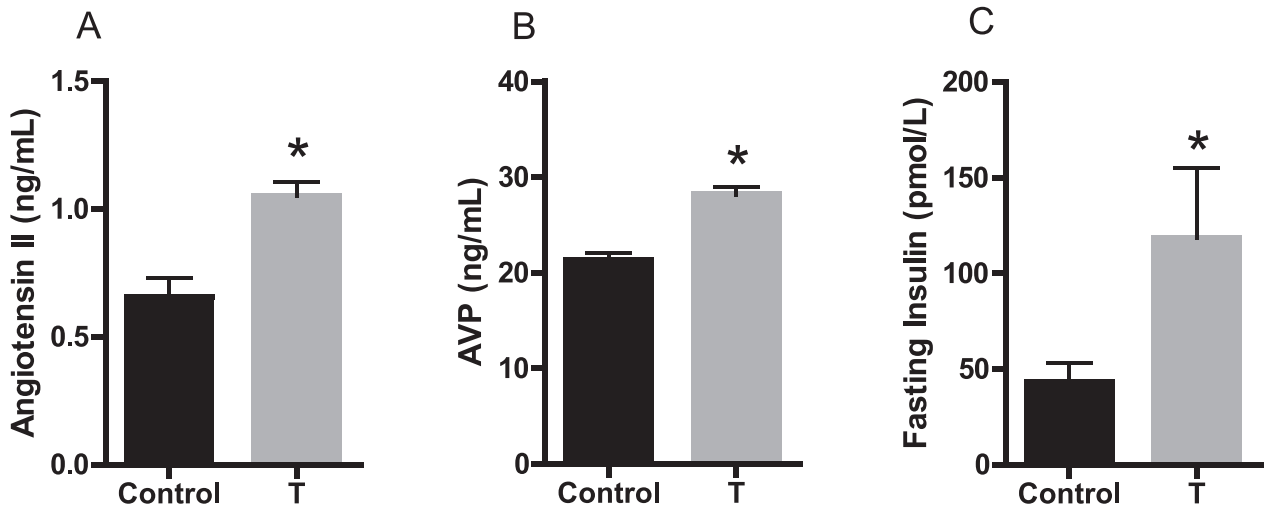

FIG. 7. Effect of prenatal T exposure on angiotensin II, AVP, and insulin levels. Angiotensin II (**A**), AVP (**B**), and insulin (**C**) levels were measured using commercially available ELISA kits. All data are expressed as mean ± SEM of six animals in each group. *$P < 0.05$ vs. control.

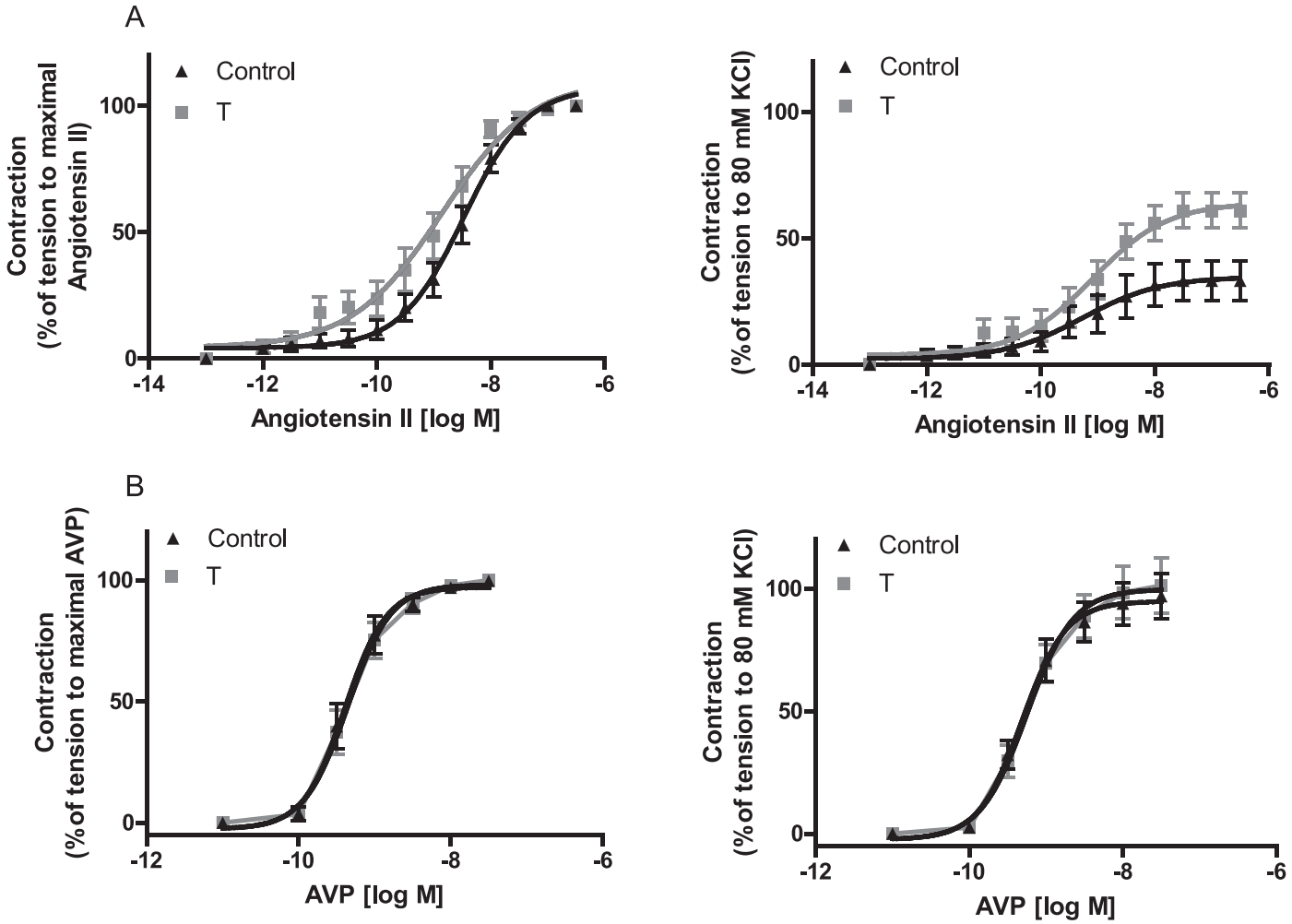

FIG. 8. Effect of prenatal T exposure on mesenteric artery responses to contractile agonists in adult females. Contractile responses were taken in endothelium-denuded mesenteric arteries to cumulative additions of angiotensin II (**A**) and AVP (**B**). Contractile responses are presented as percentage of maximal agonist contraction (left panel) and percentage of 80 mM KCl contraction (right panel) (both semilog plots). There was no significant difference in absolute tension to 80 mM KCl between prenatal T-exposed rats and controls. All data are expressed as mean ± SEM of six animals in each group.

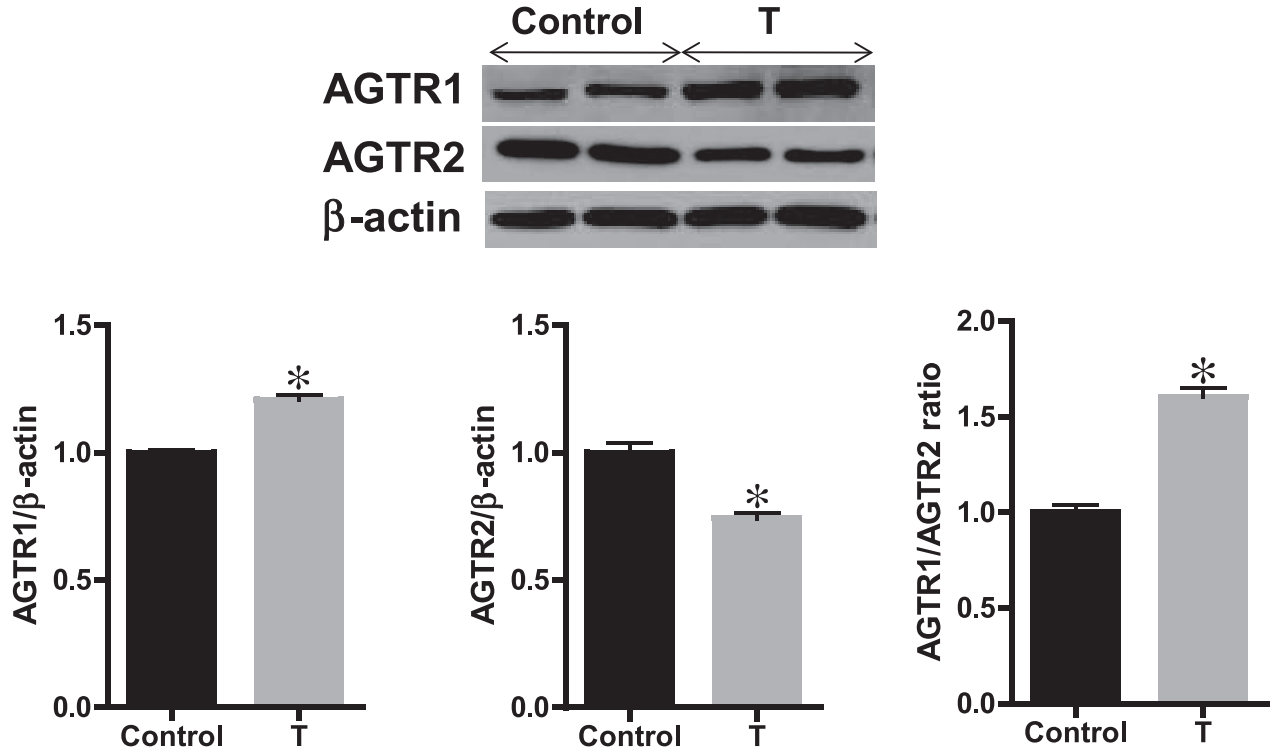

FIG. 9. Effect of prenatal T exposure on AGTR1 and AGTR2 protein expression in mesenteric arteries of adult female rats. Representative Western blots are shown at the top; blot density obtained from densitometric scanning of AGTR1 and AGTR2 normalized to β-actin and AGTR1/AGTR2 ratio is shown at the bottom. Values are presented as mean ± SEM of six animals in each group of animals. *$P < 0.05$ vs. control.

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