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Prenatal Testosterone Induces Sex-Specific Dysfunction in Endothelium-Dependent Relaxation Pathways in Adult Male and Female Rats[1]

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ABSTRACT

Prenatal testosterone (T) exposure impacts postnatal cardiovascular function, leading to increases in blood pressure with associated decreased endothelium-dependent vascular relaxation in adult females. Endothelial function in males is not known. Furthermore, which of the endothelial pathways contributes to endothelial dysfunction and if there exists sex differences are not known. The objective of this study was to characterize the relative contribution of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) to the impaired endothelium-dependent vasodilation in prenatal T-exposed adult males and females. Offspring of pregnant rats treated with T propionate or its vehicle were examined. Telemetric blood pressure levels and endothelium-dependent vascular reactivity were assessed with wire myography. Levels of nitric oxide synthase (NOS3) and Kcnn3 and Kcnn4 channel expression were examined in mesenteric arteries. Mean arterial pressure was significantly higher in T males and females than in controls. Endothelium-dependent acetylcholine relaxation was significantly lower in both T males and females. EDHF-mediated relaxation was specifically blunted in T males (E_{max} = 48.64% ± 3.73%) compared to that in control males (E_{max} = 81.71% ± 3.18%); however, NO-mediated relaxation was specifically impaired in T females (E_{max} = 36.01% ± 4.29%) compared with that in control females (E_{max} = 54.56% ± 6.37%). Relaxation to sodium nitroprusside and levcromakalim were unaffected with T-treatment. NOS3 protein was decreased in T females but not in T males. *Kcnn3* expression was decreased in both T males and females compared to controls. These findings suggest that prenatal T leads to an increase in blood pressure in the adult offspring, associated with blunting of endothelial cell-associated relaxation and that the effects are sex-specific: EDHF-related in males and NO-related in females.

blood pressure, EDHF, endothelium, Kcnn3 channels, NO, NOS3, prenatal testosterone, sex-specific, vascular function

INTRODUCTION

Cardiovascular diseases are the leading cause of mortality and morbidity in the United States. Among cardiovascular diseases, hypertension ranks first, affecting more than 73 million people, nearly 1 in 3 adults (Centers for Disease Control statistics, 2011). Recent studies indicate that adult hypertension has developmental origins (i.e., in utero exposure of the fetus to unforeseen conditions result in the development of hypertension during adult life) [1, 2]. However, the maternal factors causing programming of organ function remain unknown.

Elevated levels of androgen in pregnant mothers has been shown to cause intrauterine growth restriction and low birth weight [3–6], leading to development of adult life cardiovascular dysfunctions with enlargement of the left cardiac ventricle and kidneys [7, 8] and increase in blood pressure (BP) [4, 6, 9]. Moreover, epidemiological studies show that offspring born to pregnant polycystic ovary syndrome or preeclamptic mothers, who have elevated testosterone (T) levels [10–14], are more likely to have increased BP during postnatal life [15–18]. We have recently shown that the increases in BP in prenatal T-exposed adults were more pronounced in males than in females [6]. Furthermore, elevated BP in adult female offspring exposed prenatally to T is associated with reduced endothelium-dependent vascular relaxation involving decreased endothelial nitric oxide synthase (NOS3) expression and reduced NO production from endothelial cells [4]. The vascular changes, if any, in prenatal T-exposed adult males have not been examined.

It has been shown that endothelium plays a key role in the control of vasomotor tone and organ perfusion and contributes to the regulation of arterial BP. Prostacyclin (PGI_2), NO, and endothelium-derived hyperpolarizing factor (EDHF) are the vasodilatory factors that contribute to endothelium-dependent relaxation mechanisms [19, 20]. However, in the resistance mesenteric arteries (MA), both NO and EDHF have a major role, whereas PGI2 has a minimal role in regulating endothelium-dependent vasodilation [20, 21]. The contribution of NO and EDHF to the impaired endothelium-dependent vasodilation in the prenatal T-exposed adults is not known. Furthermore, studies examining endothelial function in animal models of developmental programming have been conducted in either males or females in isolation. Comparative examination of endothelial function in both sexes is imperative for determining potential sex-specific mechanisms of endothelial dysfunction and, thus, appropriate therapeutic strategies. The need for such studies is further justified by reports of sex differences in clinical responsiveness to antihypertensive therapies [22]. Thus, the objective of this study was to pharmacologically characterize which of the different components of endothelium-dependent dilatation contributes to the failure of endothelial function in the resistance MA of adult

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prenatal T-exposed rats and to determine if there exist sex differences in the endothelial pathways involved.

MATERIALS AND METHODS

Animals

All experimental procedures were in accordance with 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. Rats were housed in a temperature-controlled room (23°C) with a 12L:12D cycle, with food and water available ad libitum. Timed pregnancy Sprague Dawley rats (Gestational Day 12) were purchased from Harlan Laboratories, Inc. (Houston, TX). On Gestational Day 15, rats were divided into two groups; one group received daily injections of T propionate subcutaneously from Gestational Day 15 to 19 at 0.5 mg/kg/day (n = 8), and the other group received vehicle (sesame oil; n = 8). This dose and duration of exposure is commonly used to mimic plasma T levels (2-fold increase) observed in pre-eclamptic women [3, 4, 6, 20]. Dams in both groups were allowed to deliver at term, and the birth weights of pups were recorded. The number of pups in the control and T litters were adjusted to 10 pups per dam to ensure equal nutrient access for all offspring (pups with weights at each extreme were euthanized). The male:female ratio of pups remained equivalent after culling when possible. Pups were weaned at 3 wk of age, and the males and females were housed separately. At 28 wk of age, arterial pressure was monitored using a telemetry system. Following BP measurements, the animals were euthanized; MAs were separated for vascular reactivity studies, and the remaining arterial segments were quickly frozen for RNA/protein analysis.

Measurement of Blood Pressure by Radiotelemetry

Mean arterial pressure (MAP) in conscious free-moving rats was determined using a telemetry system, as we described previously [20]. 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 a week. BP levels were recorded continuously for 7 days. BP measurements obtained within a 10-sec sampling period were averaged and recorded every 10 min, 24 h a day, using software (Dataquest version 4.0; Data Sciences) provided by the manufacturer.

Small Mesenteric Artery Preparation

Rats were euthanized by CO_2 inhalation, and the mesenteric arcade was removed. Resistance MA (2-mm segments of the third-order branch of the superior mesenteric artery, 150- to 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 (mM): NaCl, 120; $NaHCO_3$, 25; KCl, 4.8; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; dextrose, 11.0; $CaCl_2$, 1.8 aerated with 95% O_2 and 5% CO_2 (pH, 7.4). 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).

Vascular Contractile Responses

The arterial rings were exposed to 80 mM potassium chloride (KCl) until reproducible depolarization-induced contractions were achieved. After a second round of washing and equilibration with Krebs buffer, vascular contractile responses to cumulative additions of phenylephrine (PE; 10^{-9} to 10^{-5} M), which causes α_1-adrenoceptor-induced contractions, were determined.

Vascular Relaxation Responses

The presence of intact endothelium in the vascular preparations was confirmed by observing the relaxation response to acetylcholine (ACh; 10^{-6} M) in rings precontracted with PE (10^{-6} M), as described previously [4]. Endothelium-dependent relaxation responses to cumulative concentrations of ACh (10^{-9} to 10^{-5} M) in rings precontracted with PE (10^{-6} M) were determined. The EDHF-mediated component of ACh vasorelaxation was assessed after inhibiting NO production with NG-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M). The NO-mediated portion of ACh relaxation was assessed after inhibiting EDHF production with charybdotoxin plus apamin (10^{-7} M each) [23]. More than one EDHF candidate exists; however, in general, the hyperpolarizing mechanism of EDHF is considered to be mediated by Kcnn3 and Kcnn4 channels (blocked by apamin and charybdotoxin, respectively) on vascular endothelium [24, 25]. All relaxation studies were done in the presence of indomethacin (10^{-5} M) to rule out any contribution of vasoactive prostanoids. Endothelium-independent relaxation responses to sodium nitroprusside (SNP; 10^{-10} to 10^{-5} M) and levcromakalim (10^{-9} to 10^{-5} M) in PE-precontracted rings were also determined.

Western Blotting

Arteries were homogenized in ice-cold RIPA buffer (Cell Signaling Technology, Danvers, MA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin. The protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 2 and 3 (Sigma Aldrich, St. Louis, MO) also were added to the homogenization buffer. Tissue lysates were centrifuged ($14\,000 \times g$ for 10 min at 4°C), and the protein content was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo Scientific, Waltham, MA). The supernatant was resuspended in NuPAGE lithium dodecyl sulfate (LDS) sample buffer and reducing agent (Invitrogen, Carlsbad, CA). Proteins (30 μg) alongside of Precision Plus Standard (Bio-Rad Laboratories, Hercules, CA) were resolved on 4%–12% gradient NuPAGE bis-Tris gels (Invitrogen) at 100 V for 2.0 h at room temperature and then transferred onto Immobilon-P membranes (Millipore Inc., Billerica, MA) at 100 V for 1.5 h. The membranes were blocked with 5% bovine serum albumin for 1 h and then incubated overnight at 4°C with primary antibodies (monoclonal NOS3, 1:500 dilution; BD Transduction Labs, San Diego, CA; or mouse monoclonal β-actin, 1:5000 dilution; Cell Signaling). After being washed, the membranes were incubated with secondary antibodies (anti-mouse conjugated with horseradish peroxidase) at 1:10 000 dilution and detected with the Pierce ECL detection kits (Thermo Scientific). Densitometric measurements were made using AlphaEase Flurochem 8000 software (Alpha Innotech, Santa Clara, CA). Results were expressed as band intensity:β-actin ratios.

Quantitative Real-Time RT-PCR

Total RNA was isolated from MA by using TRIzol reagent (Invitrogen). All RNA isolates were made DNA free by treatment with DNase and further purification with RNeasy clean-up kit (Qiagen Inc., Valencia, CA). Total RNA concentration and purity were determined using a spectrophotometer (ND-1000 model Nanodrop; Thermo Fisher Scientific, Newark, DE). Two micrograms of total RNA were reverse transcribed using a modified Maloney murine leukemia virus-derived reverse transcriptase (New England Biolabs Inc., Ipswich, MA) and a blend of oligo(dT) and random hexamer primers (Invitrogen). The reaction was carried out at 28°C for 15 min and then at 42°C for 50 min and stopped by heating at 94°C for 5 min, followed by 4°C, before storage at −20°C until further analysis. One microliter of the resulting cDNA was amplified by quantitative real-time RT-PCR (qRT-PCR) using SYBR Green (Bio-Rad) as the fluorophore in a CFX96 model real-time thermal cycler (Bio-Rad). Specific pairs of primers (*Kcnn3* forward: 5′-GCATCTCTCTGTGGATCATTGC-3′; reverse: 5′-AATCTGCTTCTCCAGGTCTTCG-3′, *Kcnn4* forward: 5′-CTACTGCACAGCAAAATCTTCACG-3′; reverse: 5′-CCTGGTATGTTTG TAGATGAGCCAC-3′; and β-*actin* forward: 5′-CGTGAAAAGATGACCCA GATC-3′; and 5′-CACAGCCTAGATGGCTACGT-3′) from published literature were used for each gene amplification. PCR conditions were 10 min at 95°C for 1 cycle, then 15 sec at 95°C, 30 sec at 60°C, and 15 sec at 72°C for 40 cycles, with 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-level increases or decreases of expression of genes of interest in prenatal T-exposed offspring versus those in control rats. All reactions were performed in duplicate, and β-*actin* was used as an internal control.

Statistics

All values are means ± SEM. Pharmacological concentration-response curves were defined by the negative log concentration that produced half of the maximum effect (pD_2) and by the maximum response (E_{max}). Sigmoidal functions were modeled to individual dose-response curves (Prism software; GraphPad Software, San Diego CA), and statistical comparisons of pD_2, E_{max}, and BP values were made by ANOVA with Bonferroni correction for multiple comparisons. For statistical comparison of single parameters, an independent *t* test was used. Statistical significance was assumed if the *P* value was <0.05. The study was powered for differences in vascular function based on previous

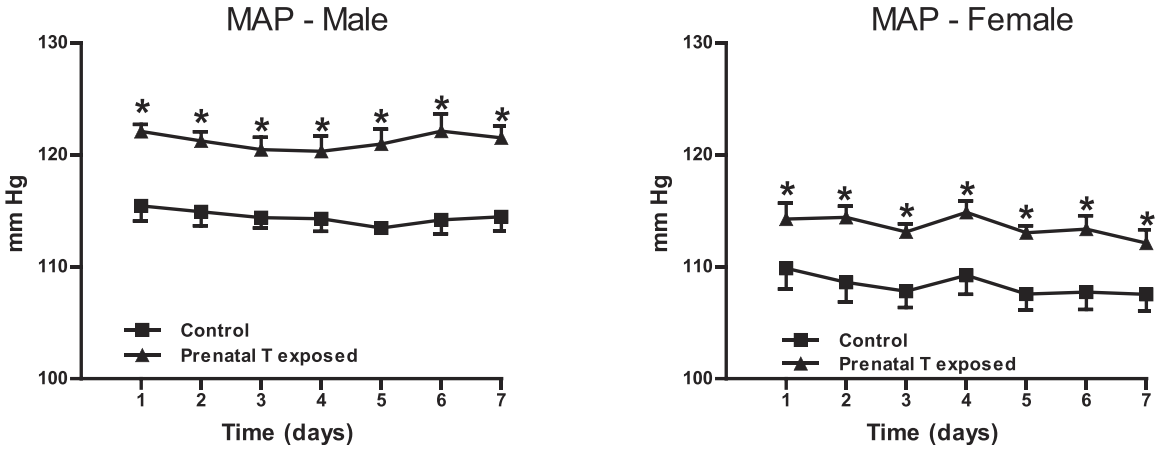

FIG. 1. Prenatal T exposure increases MAP in adult males (left panel) and females (right panel). MAP was measured 24 h per day by telemetry beginning at 28 wk of age for 7 days. Values are means ± SEM of 6–8 rats in each group. *$P < 0.05$ versus control.

studies [4, 6]. One male and one female from each litter were studied, and n refers the number of litters studied.

RESULTS

Prenatal T Exposure Increases Mean Arterial Pressure in Both Males and Females

As reported previously [4, 6] and now confirmed by telemetry, MAP was significantly higher in T males (123 ± 1.9 mm Hg; n = 7; $P \leq 0.05$) compared with control males (112 ± 0.9 mm Hg; n = 6) (Fig. 1A). MAP was also significantly higher in the T females (113 ± 1.4 mm Hg, n = 8; $P \leq 0.05$) than in control females (106 ± 1.3 mm Hg; n = 8) (Fig. 1B).

Prenatal T Exposure Does Not Alter Mesenteric Vascular Contractile Response in Both Males and Females

Vascular contractile responses to KCl, a determination of depolarization-induced vessel contraction, and cumulative doses of PE, a measurement of α_1-adrenoceptor-induced contraction, were similar in arterial segments from prenatal T-exposed males and females compared to their respective controls (Fig. 2, n = 8 in each group).

Prenatal T Exposure Impairs Endothelium-Dependent But Not Endothelium-Independent Relaxation in Both Males and Females

Endothelium-independent vascular relaxation to SNP was not different in MA from prenatal T-exposed male and females compared with respective controls (Fig. 3A). Endothelium-dependent ACh induced relaxation in PE-precontracted (10^{-6} M) arterial rings were significantly reduced in prenatal T treated males and females compared with their respective sex in controls (Fig. 3B). Sensitivity for ACh was significantly decreased in T males (pD_2 value 7.02 ± 0.12; n = 6; $P \leq 0.05$) compared to control males (pD_2 value 6.32 ± 0.12; n = 6) (Fig. 3B, left panel). ACh sensitivity was also significantly decreased in T females (pD_2 value 6.80 ± 0.11; n = 6; $P \leq 0.05$) compared to control females (pD_2 value 7.48 ± 0.05; n = 6) (Fig. 3B, right panel). These data indicate that prenatal T exposure impairs endothelium-dependent but not endothelium-independent relaxation in both male and female MA.

Prenatal T Exposure Selectively Impairs EDHF-Mediated Relaxation in Males and NO-Mediated Relaxation in Females

Consistent with our previous reports [20], simultaneous blockade of both NO and EDHF completely abolished ACh-induced vasorelaxation in MA (data not shown), indicating that NO and EDHF are the major factors responsible for responsiveness to ACh. We next characterized the contributions of NO and EDHF components in impaired ACh vasorelaxation in T offspring.

The maximal EDHF-component of ACh relaxation was significantly reduced in the T males (48.64 ± 3.73%, n = 6, $P \leq 0.05$) compared with control males (81.71 ± 3.18%, n = 6) (Fig. 4A). However, the NO component of relaxation was not significantly different between control males (41.80 ± 3.34%, n = 6) and T males (39.52 ± 2.41%; n = 6) (Fig. 4B). Vascular relaxation to levcromakalim, an ATP-sensitive K^+ channel opener used to determine the sensitivity of vascular smooth muscle cells to EDHF, was similar in MA from prenatal T-exposed males (pD_2, 6.76 ± 0.07, n = 4) compared with control males (6.60 ± 0.06, n = 4) (Fig. 4C). These data suggest that prenatal T exposure selectively impairs endothelium-dependent EDHF- but not NO-mediated relaxation in males.

In females, the maximal EDHF-mediated relaxation was not significantly different between control (80.25 ± 4.32%, n = 6) and T rats (77.38 ± 7.59%; n = 6) (Fig. 5A). In contrast, the maximal NO-mediated relaxation was significantly decreased in T females (36.01 ± 4.29%, n = 6; $P \leq 0.05$) compared with control females (54.56 ± 6.37%, n = 6) (Fig. 5B). These data suggest that prenatal T exposure selectively impairs endothelium-dependent NO- but not EDHF-mediated relaxation in females.

Prenatal T Exposure Decreases NOS3 Protein Expression in the MA of Females But Not Males

To identify the regulatory mechanism of prenatal T exposure-induced decreases in NO-mediated relaxation in females, we examined the protein levels of NOS3 in MA. The expression levels of NOS3 protein were not changed in T males (n = 5) compared with control males (n = 4) (Fig. 6, left panel). On the other hand, NOS3 protein expression was significantly decreased by 1.8-fold in T females (n = 5; $P \leq 0.05$) compared with control females (n = 4) (Fig. 6, right

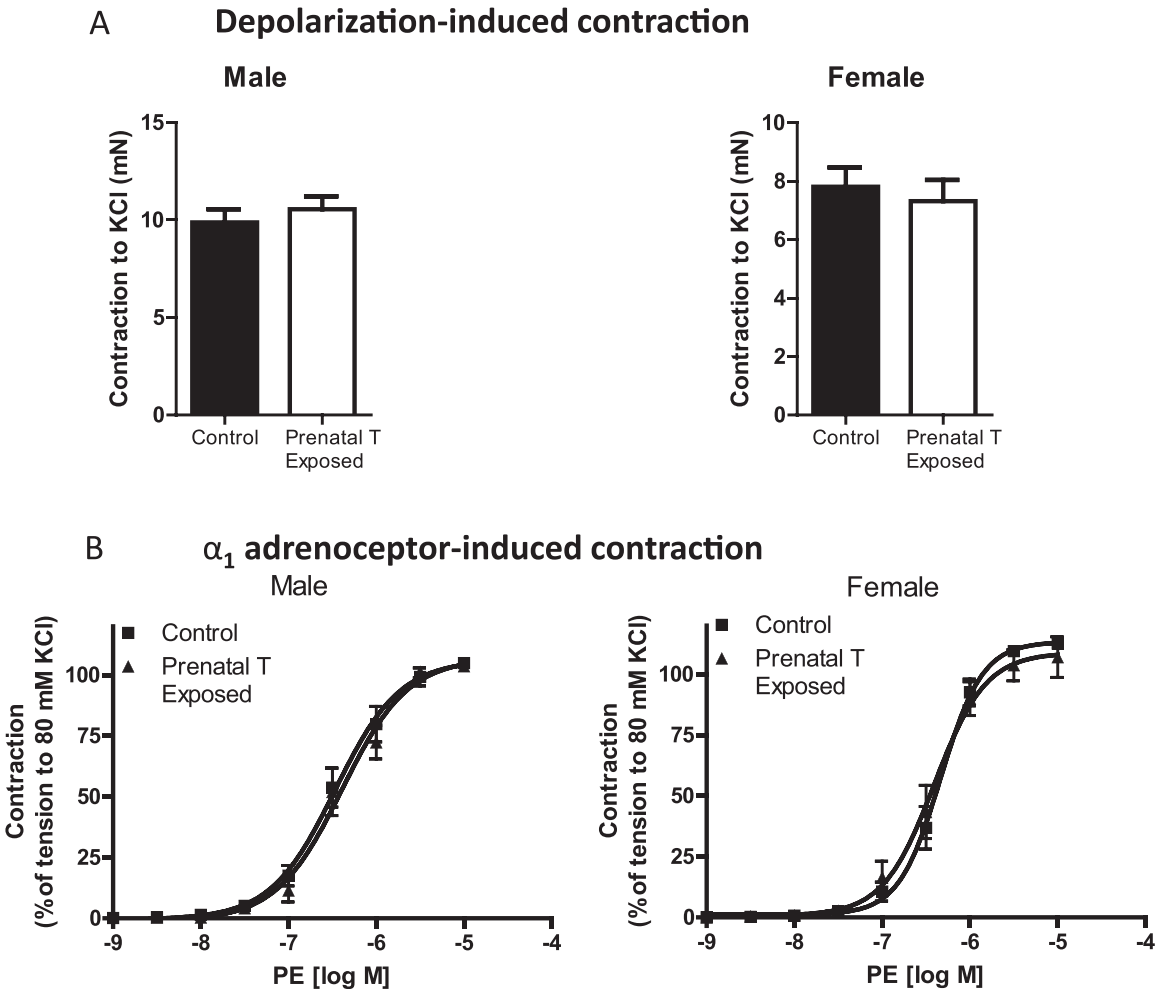

FIG. 2. Prenatal T exposure does not impair endothelium-independent vascular reactivity in MA of adult males and females. Mesenteric arterial rings were isolated from adult males and females from control and prenatal testosterone-exposed groups. Vascular contractile responses were taken to (**A**) KCl (80 mM) and to (**B**) cumulative additions of PE. Values are given as means ± SEM of 8 rats in each group.

panel). Therefore, prenatal T exposure selectively decreases NOS3 protein expression in the MA of females but not males.

Prenatal T Exposure Decreases Kcnn3 *mRNA Expression in the MA of Both Males and Females*

To identify the regulatory mechanism of prenatal T exposure-induced decreases in EDHF-mediated relaxation in males, we examined mRNA levels of the EDHF components *Kcnn3* and *Kcnn4*. *Kcnn3* expression was significantly decreased by 1.9-fold in MA from T males ($n = 8$; $P \leq 0.05$) compared with control males ($n = 8$) (Fig. 7, left panel). The expression of *Kcnn4* was not different between control and T males (Fig.7, left panel).

In females, prenatal T exposure decreased *Kcnn3* expression by 2.4-fold ($n = 8$; $P \leq 0.05$) compared with control females ($n = 8$) (Fig. 7, right panel). The expression levels of *Kcnn4* were not different between control and T females ($n = 5$) (Fig. 7, right panel). Therefore, prenatal T exposure decreased *Kcnn3* mRNA expression in MA of both males and females. Our attempt to determine Kcnn3 and Kcnn4 protein expression was less conclusive, with many bands at inappropriate molecular weights. Similar difficulties in determining Kcnn3 and Kcnn4 protein expression have been reported [26].

DISCUSSION

The major finding of this study is that maternal T levels during pregnancy elevated to clinically relevant concentrations lead to increase in BP in adult male and female offspring with more pronounced effect in males than in females. Our study demonstrates for the first time that significant gender differences exist in the endothelial relaxation pathways in adult offspring exposed to T prenatally and that this occurs through blunting of EDHF-mediated relaxation in males and impairing NO-mediated relaxation responses in females.

It is now well established that a variety of insults, when experienced in the prenatal period, can have long-term influences on the health of the individual. Of all the adverse intrauterine stimuli to which the fetus is subjected, perhaps the most important and clinically relevant is that of elevated maternal T. Higher T levels are reported in pregnant African American women [27, 28] and in several compromised pregnancies, whose offspring are at increased cardiovascular risk, such as in pre-eclampsia [10–12], maternal polycystic ovary syndrome [13, 14], protein or energy restriction [29, 30], smoking [31–37], obesity [38, 39], and stress [40, 41]. This study and our previous studies [4, 6] show that elevated T levels in pregnant rats, similar to that observed in compromised pregnancies, programs fetuses to develop increased BP during their adult lives. In many animal models, the males and females

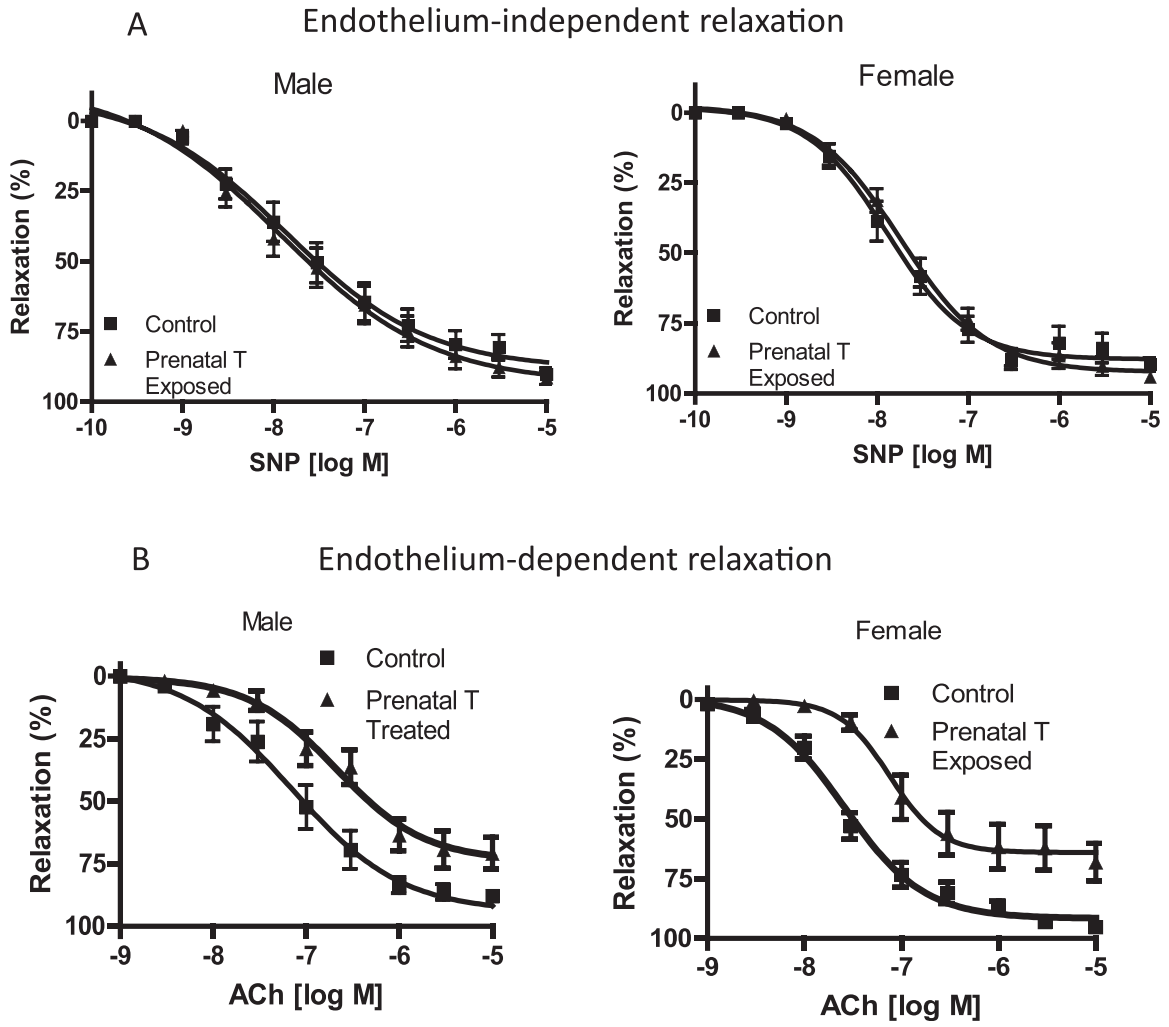

FIG. 3. Prenatal T exposure impaired endothelium-dependent vascular relaxation in MA of adult males and females. **A**) Endothelium-independent relaxation. Mesenteric arterial rings were precontracted with PE (10^{-6} M) and examined for relaxation to cumulative additions of SNP. **B**) Endothelium-dependent relaxation. Mesenteric arterial rings were precontracted with PE and examined for relaxation to ACh. Values are means ± SEM of 6 rats in each group.

are not equally affected; usually, males experience more profound effects [6, 42, 43]. In the present study, the magnitude of BP increase is different between male and female offspring of prenatally T-exposed dams, with males having greater effect than females (mean increase of 11 mm Hg in males vs. 7 mm Hg in females at 6 mo). This is consistent with our previous report of carotid arterial and tail-cuff BP measurements in prenatal T-treated rats [4, 6]. The finding that prenatal T exposure predominantly affected male offspring is in agreement with those reported in other models of programming induced by placental insufficiency [42] and maternal protein restriction [43, 44].

In order to investigate alterations of vascular function in the prenatal T-exposed adult male and female rats, responses to ACh, an endothelium-dependent vasodilator, to SNP, an endothelium-independent vasodilator, were investigated. Mesenteric vasorelaxation to ACh was reduced in both prenatal T-exposed males and females compared to respective controls, but no differences could be detected to SNP. These observations suggest that it is not the smooth muscle vasodilating capability that is reduced in prenatal T-exposed adult males and females but some function related to the endothelium. These results are consistent with previous reports of impaired endothelial function in prenatal T-exposed adult females [4].

The vascular endothelium is important in the control of vascular tone and the regulation of peripheral BP [45]. Considering that EDHF and NO are the two main factors that are responsible for endothelium-dependent relaxation in resistance arteries [20, 21], we next characterized the relative contributions of NO and EDHF to the impaired vasodilation of MA of prenatal T-exposed adult rats. Although both EDHF- and NO-mediated ACh-induced vasorelaxation mechanisms were evident in MA of control and prenatal T-exposed adults, a clear distinction between the sexes in the endothelial pathways affected was identified. Indeed, in arteries of prenatal T-exposed males the L-NAME-resistant component of ACh relaxation attributed to be EDHF was significantly reduced. In contrast, the NO component of ACh relaxation was shown to contribute equally to vasodilation in both control and prenatal T-exposed males, indicating that only the EDHF component may be impaired. This finding correlates well with previous observations that EDHF-mediated relaxation is selectively impaired in the MA of male spontaneously hypertensive rats [46, 47]. Also T is known to selectively impair EDHF-mediated relaxation in male carotid arteries [48]. In prenatal T-

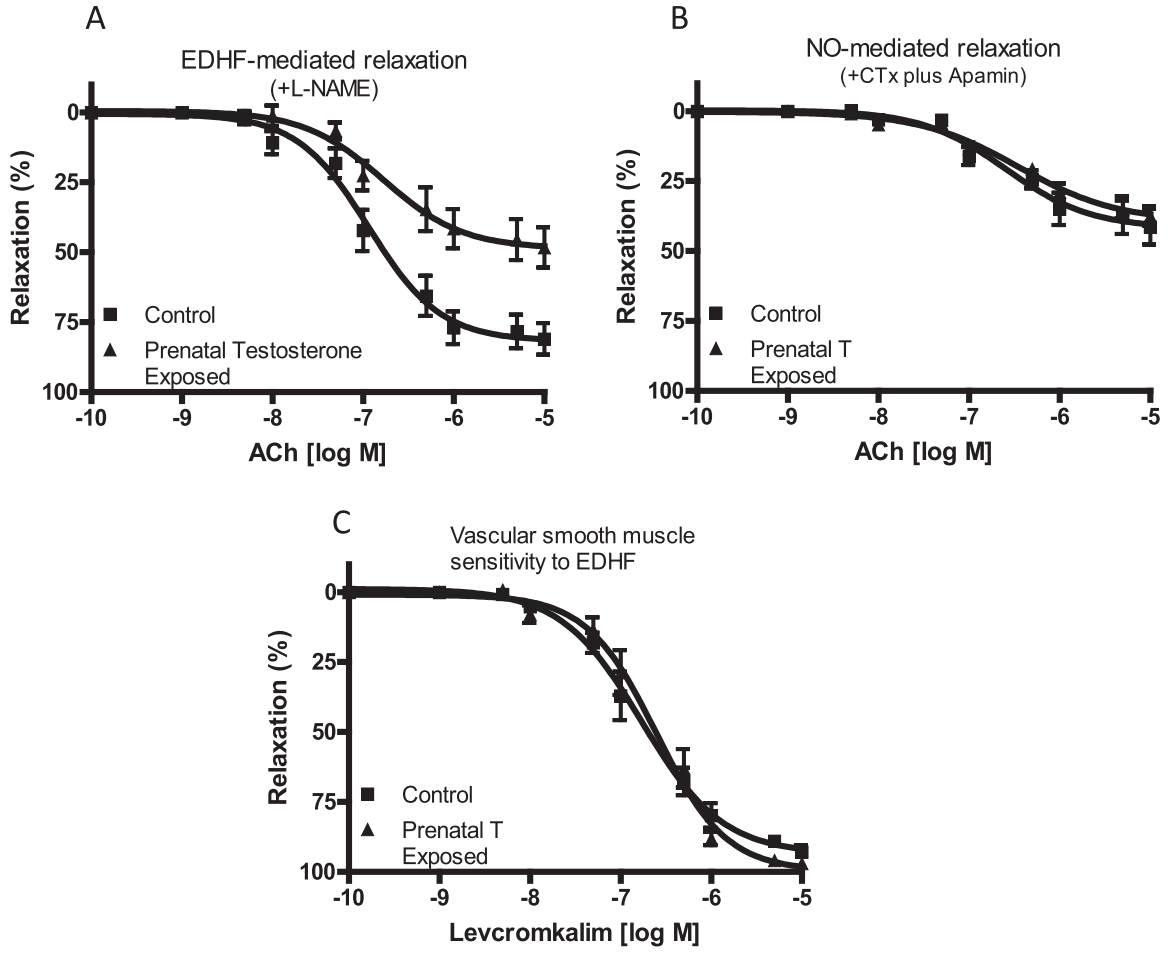

FIG. 4. Prenatal T exposure selectively impairs endothelium-dependent EDHF-mediated vascular relaxation in MA of adult males. **A**) EDHF-mediated endothelium-dependent relaxation. Mesenteric arterial rings were pretreated with the NOS inhibitor L-NAME, precontracted with PE, and examined for relaxation to ACh. **B**) NO-mediated endothelium-dependent relaxation. Mesenteric arterial rings were pretreated with the Kcnn4 blocker charybdotoxin (CTx, 10^{-7} M) and the Kcnn3 blocker apamin (10^{-7} M), precontracted with PE, and examined for relaxation to ACh. **C**) Endothelium-independent EDHF relaxation. Arterial rings were precontracted with PE and examined for relaxation to cumulative additions of levcromakalim. Values are means ± SEM (n = 6 rats; 2 vessel segments/rat).

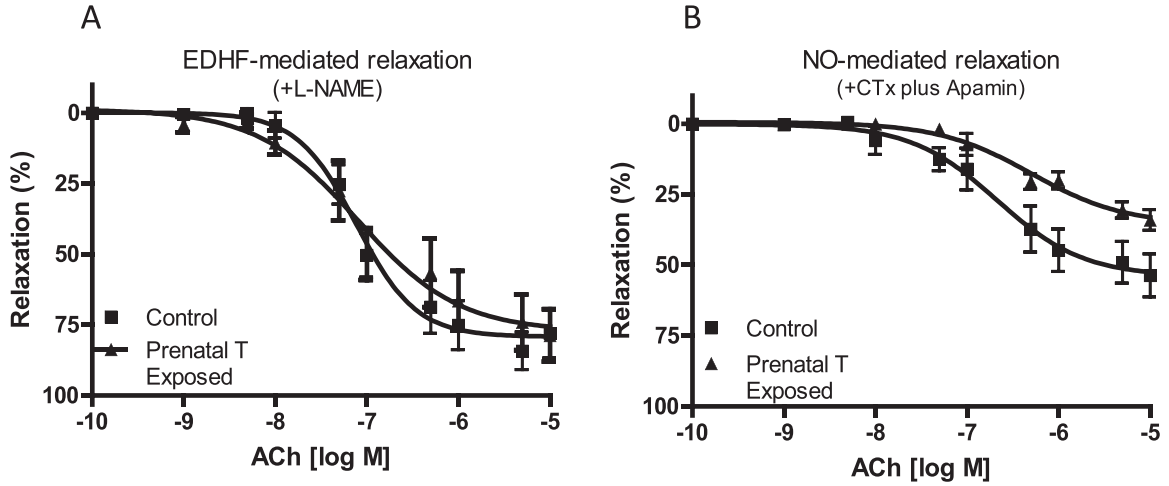

FIG. 5. Prenatal T exposure selectively impairs endothelium-dependent NO-mediated vascular relaxation in MA of adult females. **A**) EDHF-mediated endothelium-dependent relaxation. Mesenteric arterial rings were pretreated with the NOS inhibitor L-NAME, precontracted with PE, and examined for relaxation to ACh. **B**) NO-mediated endothelium-dependent relaxation. Mesenteric arterial rings were pretreated with the Kcnn4 blocker charybdotoxin (CTx, 10^{-7} M) and the Kcnn3 blocker apamin (10^{-7} M), precontracted with PE, and examined for relaxation to ACh. Values are means ± SEM (n = 6 rats; 2 vessel segments/rat).

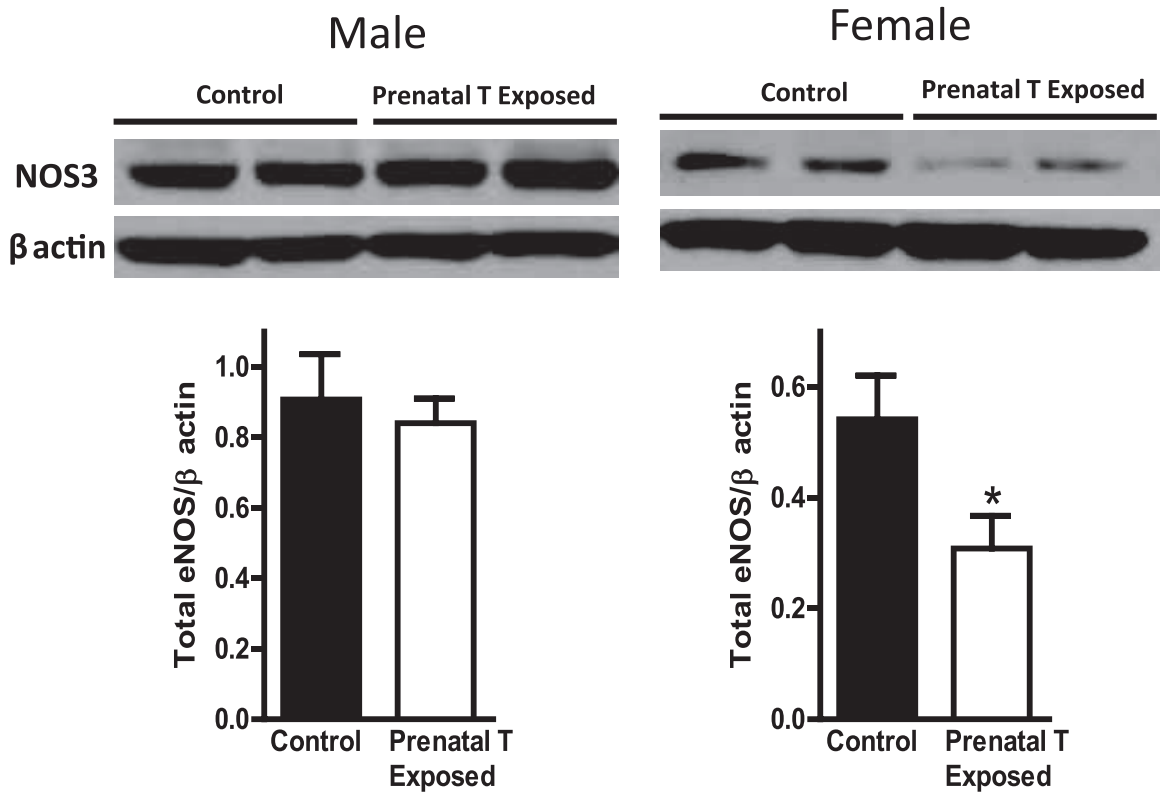

FIG. 6. Prenatal T exposure selectively decreases NOS3 protein expression in the MA of adult females. Protein were isolated from MA and probed for total NOS3. Representative Western blots for NOS3 and β-actin are shown at the top; blot density obtained from densitometric scanning of NOS3/eNOS was normalized to that of actin as shown at bottom. Values are means ± SEM of 4–5 rats in each group. *$P \leq 0.05$ versus control.

exposed males, impaired EDHF function may be due to decrease in EDHF generation from endothelial cells or may reflect a change in the sensitivity of vascular smooth muscle to relaxation by EDHF. Absence of differences in relaxation responses to levcromakalim between T and control males suggests that the decreased relaxation in T males is not due to decreased vascular smooth muscle sensitivity to EDHF but may be related to reduction in EDHF generation. Consistently, the expression *Kcnn3* channel, which is important in initiation of endothelial cell hyperpolarization, was significantly reduced in MA from the T males compared with the control males. In support of the concept that Kcnn3 channels are important for EDHF-mediated vascular function, studies using transgenic mice ($Kcnn3^{T/T}$) show that Kcnn3 channels exert a profound hyperpolarizing influence in resistance arteries and that suppression of Kcnn3 channel expression causes a pronounced and reversible hypertension [49]. Consistent with the observation of unaffected NO-mediated vasodilation, the expression of NOS3 in prenatal T-exposed males was similar to that in controls.

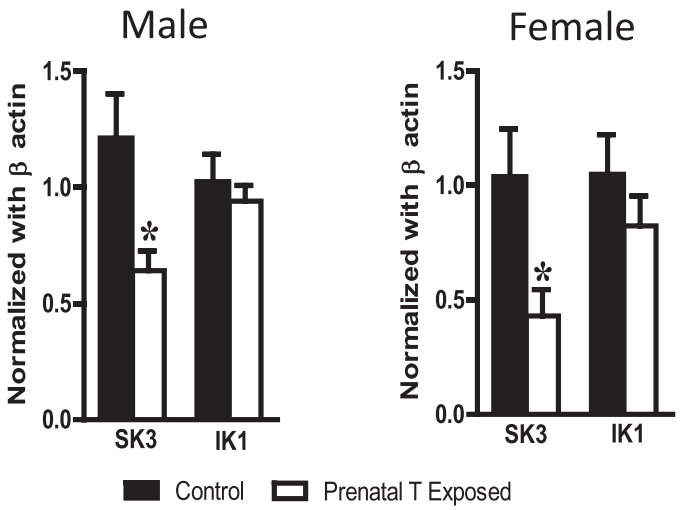

FIG. 7. Prenatal T exposure decreases *Kcnn3* channel mRNA expression in the MA of adult males and females. Real-time RT-PCR was used to assess vascular *Kcnn3/SK3* and *Kcnn4/IK1* mRNA expression levels. Quantitation of vascular *Kcnn3/SK3* and *Kcnn4/IK1* components was normalized relative to that of β-actin levels. Values are means ± SEM of 8 rats in each group. *$P \leq 0.05$ versus control.

In contrast to males, in prenatal T-exposed females, the NO component of ACh relaxation was significantly impaired compared to control females. However, the EDHF component was found to contribute equally to vasodilation in both control and prenatal T-exposed females. This finding is consistent with our observation noted in prenatal T-exposed females [4]. The decreased NO-mediated arterial relaxation in the prenatal T-exposed females is not due to decreased vascular smooth muscle sensitivity to NO, as relaxation of mesenteric rings to sodium nitroprusside, an exogenous NO donor, was not different between control and the prenatal T-exposed females. This suggests that the decreased relaxation in the prenatal T-exposed females is more likely due to changes in the synthesis/release of NO. Our data support the fact that NOS3 expression was significantly reduced in MA from the prenatal T-exposed females compared with control females. Intriguingly, the prenatal T-exposed females also have reduced expression of *Kcnn3* channels, but their EDHF responses were maintained.

This may be likely due to an augmenting effect of female sex hormones on the function of Kcnn3 channels [50].

It is interesting to know what mechanisms contribute for divergent endothelial pathways being affected in the prenatal T-exposed adult males and females. Our previous studies show that prenatal T-induced increase in postnatal T levels contributes for development and maintenance of elevated BP in both males and females [6]. One could speculate that excess androgens in adult males and females may have differential effects on the endothelium, which may contribute to the differences in the endothelial pathways affected. Indeed, sex-related differences with respect to both endothelial signaling molecule expression and endothelium-dependent vascular function have been documented [51–54]. The contribution of T, if any, to the differential and sex-specific vascular endothelial function in the prenatal T-exposed adults warrants future investigation.

It is essential to emphasize the following cautionary remarks regarding the aforesaid interpretations. First, although the decrease in endothelial cell function contributes to the observed elevation in arterial pressure, the endothelial dysfunction may also be secondary to arterial pressure alterations. Further analysis of longitudinal changes in endothelial function and BP are essential to establish a cause-effect relationship. Second, other factors such as neuronal and renal mechanisms can also facilitate or contribute to vascular dysfunction. The possible additional alterations in other systems represent important areas for future experiments.

In summary, increase in T levels in pregnant rats, similar to that observed in compromised pregnancies, programs fetuses to develop hypertension during their adult lives, with a more pronounced effect in males than females. These hypertensive progenies have impairments in mesenteric vascular endothelial function in a sex-dependent manner: selective impairments in EDHF-mediated vasodilator pathway in males and NO-mediated relaxation system in females. The decreased endothelium-dependent vascular relaxation pathway involving reduced release of EDHF or NO from endothelial cells may play an important role to contribute at least in part for the increased arterial pressure in prenatal T-exposed adults.

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