Elevated Testosterone Levels During Rat Pregnancy Cause Hypersensitivity to Angiotensin II and Attenuation of Endothelium-Dependent Vasodilation in Uterine Arteries

Vijayakumar Chinnathambi, Chellakkan S. Blesson, Kathleen L. Vincent, George R. Saade, Gary D. Hankins, Chandra Yallampalli, Kunju Sathishkumar

Abstract—Elevated testosterone levels increase maternal blood pressure and decrease uterine blood flow in pregnancy, resulting in abnormal perinatal outcomes. We tested whether elevated testosterone alters uterine artery adaptations during pregnancy, and whether these alterations depend on endothelial-derived factors such as nitric oxide, endothelium-derived hyperpolarizing factor, and prostacyclin, or endothelium-independent mechanisms such as angiotensin II (Ang-II). Pregnant Sprague–Dawley rats were injected with vehicle (n=20) or testosterone propionate (0.5 mg/kg per day from gestation day 15 to 19; n=20). Plasma testosterone levels increased 2-fold in testosterone-injected rats compared with controls. Elevated testosterone significantly decreased placental and pup weights compared with controls. In endothelium-intact uterine arteries, contractile responses to thromboxane, phenylephrine, and Ang-II were greater in testosterone-treated rats compared with controls. In endothelium-denuded arteries, contractile responses to Ang-II (pD2=9.1±0.04 versus 8.7±0.04 in controls; P<0.05), but not thromboxane and phenylephrine, were greater in testosterone-treated rats. Ang-II type 1b receptor expression was increased, whereas Ang-II type 2 receptor was decreased in testosterone-exposed arteries. In endothelium-denuded arteries, relaxations to sodium nitroprusside were unaffected. Endothelium-dependent relaxation to acetylcholine was significantly lower in arteries from testosterone-treated dams (E_max=51.80±6.9% versus 91.98±1.4% in controls; P<0.05). The assessment of endothelial factors showed that nitric oxide–, endothelium-derived hyperpolarizing factor–, and prostacyclin-mediated relaxations were blunted in testosterone-treated dams. Endothelial nitric oxide synthase, small conductance calcium–activated potassium channel-3, and prostacyclin receptor expressions were significantly increased in arteries from testosterone-treated dams. Hypoxia-inducible factor-1α, Ankr37, and Egln were significantly increased in testosterone-exposed placentas. These results suggest that elevated maternal testosterone impairs uterine vascular function, which may lead to an increased vascular resistance and a decrease in uterine blood flow. (Hypertension. 2014;64:405-414.) ● Online Data Supplement

Key Words: endothelium-dependent hyperpolarization factor ♦ fetal development ♦ nitric oxide ♦ prostacyclin ♦ uterine artery ♦ vasoconstriction ♦ vasodilation

Elevated maternal testosterone levels have long been associated with adverse pregnancy outcomes, for the mother, her fetus, and the newborn. Increased maternal testosterone produces at least 2 major potential effects: direct actions of testosterone on the fetus and indirect actions on the maternal–fetal unit, which result in intrauterine growth retardation and chronic conditions in adult life, such as hypertension, dyslipidemia, and diabetes mellitus.1–6 Numerous studies have demonstrated that testosterone directly causes fetal damage,7–11 whereas the adverse effects of testosterone on fetal growth could be from indirect action of testosterone on the maternal–fetal unit and the uteroplacental circulation. Studies in humans have shown that elevated testosterone as observed in polycystic ovary syndrome pregnancies is associated with impaired decidual trophoblast invasion, increased uterine artery resistance index, and reduced blood flow, resulting in an increase in abnormal perinatal outcomes.12,13 However, the mechanisms underlying the testosterone-induced reduction of uterine blood flow in pregnancy are not fully understood.

During pregnancy, the development of uteroplacental circulation with low vascular tone accommodates >20-fold increase in uterine blood flow in near-term pregnant sheep and in humans, which ensures normal placental perfusion and fetal development.14,15 The adaptation of uterine artery...
contraction and relaxation mechanisms to pregnancy is complex. In addition to growth and remodeling of the uterine vasculature, decreased uterine artery resistance is accomplished by significantly blunted vascular contractility and increased endothelial nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) synthesis/release with enhanced endothelium-dependent relaxation of the uterine artery. The effects of testosterone on vascular contractions are controversial and vary in different species, sex, vascular bed, and experimental conditions. Testosterone can inhibit NO, prostacyclin, and EDHF production or function and decrease endothelium-dependent vasorelaxation. In addition, both vasoconstriction and relaxation induced by testosterone have been reported previously. Recent studies in rat models also suggest an important role for androgens in inducing key features of preeclampsia, including elevated mean arterial pressure, proteinuria, systemic endothelial dysfunction, and reduced fetal weight. It is also demonstrated that increased testosterone levels are associated with impaired placental development with significant decrease in endovascular trophoblast invasion and nutrient transport capacity. However, despite the well-documented effects of elevated maternal testosterone in placental development and the available circumstantial evidence of its association with increased uterine artery resistance and pulsatility index and decreased uterine blood flow in polycystic ovary syndrome pregnancy, the effect of testosterone on uterine artery contractility has not been studied. Herein, we present evidence in an in vivo pregnancy rat model system that elevated testosterone, at concentrations similar to that observed in clinical conditions, promotes selective hyper-responsiveness to angiotensin II (Ang-II) and impairs endothelium-dependent NO-, EDHF-, and prostacyclin-mediated relaxation in uterine arteries associated with placental hypoxia.

Materials and Methods

All procedures were in accordance with National Institutes of Health guidelines (NIH Publication 85-23, revised 1996) with approval by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch at Galveston. On gestational day 13, dams were randomly divided into 2 groups. Dams in the treatment group were subcutaneously injected with testosterone propionate (0.5 mg/kg per day; n=20) for 5 days from gestational day 15 to 19. The control group received vehicle (sesame oil; n=20). This dose and duration of exposure is commonly used to mimic the 2-fold increase in plasma testosterone levels observed in preeclamptic women and polycystic ovary syndrome pregnancies. At gestational day 20, animals were euthanized, a portion of the uterine arteries was separated for vascular reactivity studies, and the remaining arteries and placenta were quickly frozen for RNA/protein isolation. Fetal weight and crown-rump length and placental weight and diameter were measured. Some dams were allowed to deliver at term, and the birth weight of pups was recorded. An expanded Methods section is available in the online-only Data Supplement, which includes animals, arterial segment preparation, vascular contractile and relaxation responses, RNA isolation and quantitative real-time polymerase chain reaction, Western blotting, and statistical analysis.

Results

Elevated Testosterone Decreased Placental Weights and Birth Weight of Pups

The length of gestation and mean litter size were not significantly affected by testosterone treatment (Table S2 in the online-only Data Supplement). Fetal weight and crown-rump length, placental weight, and birth weight of pups were significantly reduced in the testosterone-treated group compared with controls (Table S2), consistent with previous reports. The plasma testosterone levels in testosterone-treated dams were 2.2±0.23 ng/mL compared with 1.0±0.25 ng/mL in vehicle-treated control dams (n=7 in each group; P<0.05).

Figure 1. Testosterone (T) exposure enhances uterine artery responses to contractile agonists. Contractile responses were taken in endothelium-intact (Endo+) and endothelium-denuded (Endo−) uterine arteries to cumulative additions of (A) thromboxane agonist U46619, (B) phenylephrine (PE), and (C) angiotensin II (Ang-II). D, Ang-II dose response in Endo+ arteries in absence or presence of losartan and PD123319 (PD). Values are given as mean±SEM of 5 to 8 rats in each group.
**Contractile Response to Ang-II Was Selectively Increased in Endothelium-Denuded Uterine Arteries of Testosterone-Treated Rats**

Figure 1 shows the effect of elevated testosterone exposure on U46619-, phenylephrine-, and Ang-II–induced concentration-dependent contractions of endothelium-intact and endothelium-denuded uterine arteries. As shown in the Table, in endothelium-intact arteries, the maximal response and the pD2 values of U46619-, phenylephrine-, and Ang-II–induced contractions were significantly increased in testosterone-treated rats compared with controls (n=5–8 in each group; P<0.05). Removal of the endothelium significantly enhanced U46619-, phenylephrine-, and Ang-II–induced contraction to a greater extent in control than in testosterone-treated rats (Figure 1 and Table; n=7–8 in each group; P<0.05). The U46619- and phenylephrine-induced contractions in endothelium-denuded arteries of testosterone-treated rats were not significantly different from controls (Figure 1A and 1B and Table; n=5–8 in each group). In contrast, in endothelium-denuded arteries, there remains a significant increase in Ang-II–induced contraction in testosterone-treated rats, as compared with that of controls (Figure 1C and Table; n=8 in each group; P<0.05). These data indicate that testosterone selectively increases Ang-II–induced contraction in endothelium-denuded uterine arteries.

**Losartan and PD123319 on Ang-II–Induced Constrictions**

To determine the receptor subtype through which Ang-II mediated vascular contractions, uterine arterial rings were pretreated with losartan or PD123319. Losartan completely blocked Ang-II–induced contractions of the endothelium-intact and endothelium-denuded arteries from both control and testosterone-treated rats (Figure 1D and Table; n=5–8 in each group). PD123319 significantly enhanced Ang-II–induced contractions in endothelium-intact arteries of both control and testosterone-treated rats; however, the magnitude of increase was greater in the arteries of controls than in testosterone-treated rats (Figure 1D and Table; P<0.05; n=5–8 in each group). PD123319 did not significantly affect Ang-II–induced contractions in endothelium-denuded arteries from control and testosterone-treated rats (Figure S3).

**Uterine Arterial Expression of Ang-II Receptors in Testosterone-Treated Rats Is Altered With Increased Ang-II Type 1b Receptor and Decreased Ang-II Type 2 Receptor Levels**

To determine whether Ang-II receptor (ATR) expression in the uterine arteries correlated with alteration of Ang-II contractile responses in testosterone-treated rats, mRNA and protein levels of ATRs were determined with quantitative reverse transcriptase polymerase chain reaction and Western blot analyses. As shown in Figure 2A, testosterone-treated rats had no significant changes in Ang II type-1a receptor (AT1aR) mRNA expression, but significantly increased Ang II type-1b receptor (AT1bR) mRNA expression by 3-fold in uterine arteries compared with controls (n=6 in each group; P<0.05). In contrast, AT1R was significantly decreased by 50% in uterine arteries of testosterone-treated rats (n=6) compared with controls (n=6; P<0.05; Figure 2A). Thus, the presence of elevated testosterone levels significantly increased the AT1bR/AT1R ratio in uterine arteries by 6-fold compared with controls (n=6; P<0.05; Figure 2A).

There are no commercial antibodies available that can individually detect AT1aR and AT1bR; hence we used an antibody that detects both isoforms. As shown in Figure 2B, testosterone-treated rats showed a trend of increase in AT1R protein expression in uterine arteries compared with controls (n=6; P=0.06; Figure 2B). However, AT1R protein was significantly decreased in uterine arteries of testosterone-treated rats compared with controls (n=6 in each group; P<0.05; Figure 2B). The AT1R/AT1aR ratio in uterine arteries was significantly higher by 2.1-fold in testosterone-treated rats compared with controls (n=6; P<0.05; Figure 2B).

**Endothelium-Dependent, but Not Endothelium-Independent, Relaxation in Uterine Arteries Is Impaired in Testosterone-Treated Rats**

We next determined the effect of testosterone exposure on endothelium-dependent relaxations induced by acetylcholine.
in uterine arteries. As shown in Figure 3A, acetylcholine produced a concentration-dependent relaxation in uterine arteries from both control and testosterone-treated rats. Testosterone exposure resulted in a significant decrease in the maximal relaxation induced by acetylcholine (control: 91.98±1.4%; testosterone-treated rats: 51.80±6.9%; n=9 in each group; P<0.05).

To determine the potential effect of testosterone exposure on endothelium-independent relaxations, sodium nitroprusside (SNP)-induced relaxations of the uterine arteries were also examined in the present study. SNP-induced relaxations of the uterine arteries were not significantly different between control and testosterone-treated rats (Figure S4; n=9 in each group). These data indicate that testosterone impairs endothelium-dependent, but not endothelium-independent, relaxation in uterine arteries.

NO-, EDHF-, and Prostacyclin-Mediated Endothelium-Dependent Relaxation Is Decreased in Uterine Arteries of Testosterone-Treated Rats

Because endothelium-dependent relaxation is mediated by NO, EDHF, and prostacyclin, we next examined the effect of elevated testosterone on these 3 distinct relaxation pathways. The NO component of relaxation was significantly reduced by 33% in the testosterone-treated rats (E_max=48.64±3.73%; n=8; P<0.05) compared with controls (E_max=72.56±11.51%; n=8; Figure 4A). The EDHF component of acetylcholine-induced relaxation was significantly reduced by 84% in testosterone-treated rats (E_max=9.72±8.76%; n=8; P<0.05) compared with controls (E_max=62.33±3.70%; n=7; Figure 4B). The maximal prostacyclin-mediated relaxation was 50.18±8.53% (n=8; P<0.05) in control rats, and this was completely abolished in testosterone-treated rats (−3.18±1.73%; n=8; Figure 4C). Blockade of all 3 pathways completely abolished acetylcholine-induced relaxation (n=7; Figure 4D). These data suggest that presence of elevated testosterone levels in pregnant rats impairs all 3 endothelial relaxation pathways.

Endothelial NO Synthase, Small Conductance Calcium–Activated Potassium Channel-3, and Prostacyclin Receptor Expression Is Decreased in Uterine Arteries of Testosterone-Treated Rats

To determine whether endothelial NO synthase (eNOS) expression in the uterine arteries correlated with decrease of NO-mediated relaxations in testosterone-treated rats, eNOS expression and activity status was determined. There was a significant decrease in eNOS mRNA (Figure 5A) and protein levels (Figure 5B) in uterine arteries from testosterone-treated rats (n=6; P<0.05) compared with controls (n=6).
Examination of phosphorylation status of eNOS as an indicator activity state showed that phosphorylation at Ser1177 was significantly lower in uterine arteries of testosterone-treated rats compared with controls (Figure 5B; P≤0.05; n=5 in each group).

Small conductance calcium–activated potassium channel-3 (SK3) and intermediate conductance calcium–activated potassium channel-1 (IK1) are a major source of EDHF generation in the vasculature. We determined whether the expression of SK3 and IK1 in the uterine arteries correlated with decrease of EDHF-mediated relaxations in testosterone-treated rats. There was a significant decrease in SK3 mRNA levels in uterine arteries from testosterone-treated rats (n=6; P≤0.05) compared with controls (n=6; Figure 6, left panel). However, the mRNA levels of IK1 were not significantly different between control and testosterone-treated rats (n=6 in each group; Figure 6). Our attempt to determine SK3 and IK1 protein expression was less conclusive with many bands at inappropriate molecular weights similar to previous reports.38

Studies show that prostacyclin is synthesized through cyclooxygenase-2 and prostacyclin synthase and acts on prostacyclin receptor to cause vasodilation. The expression levels of cyclooxygenase-2, prostacyclin synthase, and prostacyclin receptor in uterine arteries were determined to find whether they correlated with decrease of prostacyclin-mediated relaxations in testosterone-treated rats. There was no significant difference in the mRNA and protein levels of cyclooxygenase-2 and prostacyclin synthase, but the prostacyclin receptor expression (n=6; P≤0.05) was significantly reduced in uterine arteries of testosterone-treated rats compared with controls (n=6; Figure 7A and 7B).

Expression of Hypoxia Responsive Genes Is Increased in Placentas of Testosterone-Treated Rats
Enhanced uterine artery contraction and reduced relaxation mechanisms may decrease uterine arterial blood flow, which would be anticipated to decrease oxygen delivery to the placenta. We, therefore, predicted that the placentas in testosterone-treated rats would be more hypoxic in vivo. To test this, we examined the expression of hypoxia-inducible factor-1α (HIF-1α, a key transcription factor for response to low oxygen) and other hypoxia responsive genes, Ankrd37 and Egln. The mRNA and protein levels of HIF-1α were significantly increased in placentas of testosterone-treated rats (n=6; P≤0.05) compared with control placentas (n=6; Figure 8A). Consistently, the measurement of hypoxia responsive genes, Ankrd37 and Egln, also showed a significant increase in their mRNA levels in the placentas of testosterone-treated rats (n=6; P≤0.05) compared with control placentas (n=6; Figure 8B).

Discussion
The major findings of the present study are that in pregnant rat uterine arteries, a 2-fold increase in plasma testosterone levels, similar to that observed in compromised pregnancies, (1) did not affect thromboxane- and α1-adrenoceptor–mediated contractions, but concentration-dependently increased AngII–induced contractions with associated increase in AT1R expression and decrease in AT2R expression, and (2) did not affect endothelium–independent relaxations mediated by SNP, whereas attenuated the acetylcholine-induced, endothelium–dependent NO−, EDHF−, and prostacyclin–mediated relaxations with correlated decreases in eNOS, SK3, and prostacyclin receptor expression, respectively. The enhanced uterine artery contraction and reduced relaxation
Testosterone (T) exposure decreases small conductance calcium–activated potassium channel-3 (SK3) mRNA expression in uterine arteries. Real-time reverse transcriptase polymerase chain reaction was used to assess vascular SK3 and conductance calcium–activated potassium channel-1 (IK1) mRNA expression. Quantification of vascular SK3 and IK1 components was normalized relative to β-actin levels. Values are given as mean±SEM of 6 rats in each group. *P≤0.05 vs control.
have been reported between mesenteric and uterine vascular beds. Indeed, major differences in vascular bed are present and its phosphorylation status at Ser 1177 in uterine arteries of testosterone-treated rats suggests that elevated testosterone levels significantly decreased plasma NOx levels, further emphasizing that testosterone decreases the function of the eNOS enzyme.

The present finding of decreased EDHF-mediated relaxation in uterine arteries of testosterone-treated rats correlates well with the decreased expression of SK3, which is important in the initiation of endothelial cell hyperpolarization. These findings agree well with previous studies that showed a testosterone-dependent reduction in SK3 expression in rat mesenteric arteries. In support of the concept that SK3 is important for EDHF-mediated vascular function, studies using transgenic mice (SK3<sup>T/T</sup>) show that SK3 exerts a profound hyperpolarizing influence in resistance arteries and that suppression of SK3 expression causes decreased vasodilation and pronounced hypertension. A possible mechanism for decreased prostacyclin-mediated vasodilation is via inhibition of cyclooxygenase or prostacyclin synthase, which are involved in prostacyclin production. Previous studies show that testosterone inhibits prostacyclin production in cultured aortic vascular smooth muscle cells in vitro. Although we did not measure prostacyclin production in this study, it is less likely that prostacyclin production is altered in uterine arteries of testosterone-treated rats because similar expression levels of prostacyclin biosynthetic enzymes were observed in testosterone-treated and control rats. However, the expression of prostacyclin receptor that mediates the vasodilatory effects of prostacyclin was significantly reduced in uterine arteries of testosterone-treated rats, suggesting that decreased prostacyclin receptor may contribute to reduced prostacyclin mediated vasodilation.

The present study shows that placentas from testosterone-treated rats have significantly increased expression of HIF-1α, suggesting that decreased prostacyclin receptor may contribute to reduced prostacyclin mediated vasodilation.

**Figure 7.** Testosterone (T) exposure decreases prostacyclin (IP) receptor (IPR) mRNA and protein expression in uterine arteries. A, mRNA expression. Real-time reverse transcriptase polymerase chain reaction was used to assess vascular cyclooxygenase-2 (Cox-2), prostacyclin synthase (Pgis), and IPR mRNA expression. Quantification of vascular Cox-2, Pgis, and IPR was normalized relative to β-actin levels. B, Protein expression. Proteins were isolated from uterine arteries and probed for Cox-2, Pgis, and IPR. Representative Western blots for Cox-2, Pgis, IPR, and β-actin are shown at bottom. Values are given as mean±SEM of 6 rats in each group. <sup>*</sup>P<0.05 vs control.
cause fetal growth restriction are also presented with high androgen levels, such as preeclampsia, polycystic ovary syndrome, congenital adrenal hyperplasia, maternal smoking or nicotine intake, caffeine intake, obesity, or stress. Moreover, pregnant black women have higher serum testosterone levels, with a greater frequency of low-birth-weight babies. Thus, it is of clinical significance to examine androgen’s role in fetal growth restriction. The present study demonstrates, for the first time to our knowledge, that elevated testosterone levels have adverse effects on vascular reactivity of the uterine artery in pregnancy with selective increase in Ang-II–mediated contraction and decreased endothelium-dependent relaxations. Although it is not clear at present whether increased vasoconstriction and inhibition of endothelium-dependent relaxation could be a major reason for reduced uterine blood flow and fetal nutrient delivery observed with androgen exposure during pregnancy, these findings provide a potential mechanism. Strategies that target excessive androgen action in uterine circulation could have important therapeutic potential in treatment of pregnancies complicated by fetal growth restriction.

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Disclosures

None.

References


What Is New?

• In contrast to the well-studied beneficial roles of estrogen and progesterone in maternal cardiovascular adaptations to pregnancy, this study shows that elevated maternal plasma testosterone, at levels similar to those observed in preeclampsia, polycystic ovary syndrome mothers, and pregnant black women, leads to enhanced vascular contraction and blunted endothelium-dependent relaxation in uterine arteries of pregnant rats.

• Testosterone-mediated increases in uterine arterial contraction are mediated by selective hyper-responsiveness to angiotensin II, which correlates with increased angiotensin II type 1b receptor expression and decreased angiotensin II type 2 receptor expression.

• Testosterone-mediated impairments in uterine arterial relaxation are mediated by decreases in endothelium-dependent nitric oxide, endothelium-derived hyperpolarizing factor, and prostacyclin components.

• The enhanced uterine artery contraction and reduced relaxation mechanisms may decrease uterine arterial blood flow that may lead to decreased oxygen delivery to the placenta as indicated by an increase in the expression of hypoxia responsive genes in placentas.

What Is Relevant?

• Most pregnancy pathologies, which cause fetal growth restriction, are also presented with high androgen levels, such as preeclampsia, polycystic ovary syndrome, congenital adrenal hyperplasia, maternal smoking or nicotine intake, caffeine intake, obesity, or stress. Moreover, pregnant black women have higher serum testosterone levels, with a greater frequency of low-birth-weight babies. Thus, it is of clinical significance to examine androgen’s role in fetal growth.

• Numerous studies have demonstrated that testosterone directly causes fetal damage, whereas the adverse effects of testosterone on fetal growth could be from indirect action of testosterone on the maternal–fetal unit and the uteroplacental circulation. Herein, we present evidence that elevated testosterone levels have adverse effects on vascular reactivity of the uterine artery in pregnancy with selective increase in angiotensin II-mediated contraction and decreased endothelium-dependent relaxations.

• The increased vasoconstriction and inhibition of endothelium-dependent relaxation could be a major reason for reduced uterine blood flow and fetal nutrient delivery observed with androgen exposure during pregnancy.

• Strategies that target excessive androgen action in uterine circulation could have important therapeutic potential in treatment of pregnancies complicated by fetal growth restriction.

Summary

This article is the first to show how elevated maternal testosterone, at concentrations relevant to those observed in abnormal pregnancy conditions such as preeclampsia, affects uterine artery function. Elevated testosterone enhanced uterine artery vasoconstriction and blunted endothelium-dependent relaxation, which may lead to increased vascular resistance and a decrease in uterine blood flow.
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Elevated testosterone levels during rat pregnancy causes hypersensitivity to ANG II and attenuation of endothelium-dependent vasodilation in uterine arteries

Vijayakumar Chinnathambi, Chellakkan S. Blesson, Kathleen L. Vincent, George R. Saade, Gary D. Hankins, Chandra Yallampalli, and Kunju Sathishkumar*

1Division of Reproductive Endocrinology, Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston, TX 77555, 2Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas 77030

Short Title: Elevated Testosterone Alters Uterine Vascular Function
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*Corresponding author and reprint requests:
K. Sathishkumar, DVM, PhD
Assistant Professor, Dept. of Obstetrics & Gynecology
University of Texas Medical Branch
301 University Blvd.
Galveston, TX 77555-1062
Phone: (409) 772-7592 Fax: (409) 772-2261
Email: kusathis@utmb.edu
**Methods:**

**Animals**

Timed pregnant Sprague-Dawley rats (day 12 of gestation; copulation plug on day 1; Charles River, Wilmington, MA) were used in the experiment. Rats were housed in polypropylene cages in a temperature and humidity-controlled environment. Animal housing facilities were controlled on a 12-hour light, 12-hour dark cycle and rats were given free access to standard laboratory chow and water.

**Arterial segment preparation**

Rats were sacrificed by CO₂ asphyxiation on GD20, the uterine horn was removed by laparotomy and the tissue was placed directly into ice-cold Krebs buffer (in mM: NaCl, 119; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.17; NaHCO₃, 25; KH₂PO₄, 1.18; EDTA, 0.026; and d-glucose, 5.5; pH 7.4). Under a dissecting microscope, the main uterine artery, at the midpoint of the uterine arcade, was identified and dissected free from fat and connective tissue. Uterine vessels were cut into 1.5 mm segments and two 25 µm wires were threaded through the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer, while the other was attached to an isometric force transducer (Multi Myograph, Model 610M Danish Myo Technologies, Aarhus, Denmark). The myograph organ bath (6 mL) was filled with Krebs buffer maintained at 37°C and aerated with 95% O₂–5% CO₂. The diameter of the vessels was determined by placing the wire myograph onto the stage of inverted light microscope fitted with 40x objective and calibrated graticule. The inner diameter that corresponds to the outer edge of the two wires was determined by holding the vessels under just enough tension to have the vessel wall separated. The vessels were then washed and incubated for 30 min under zero force before performing the normalization procedure. Each arterial segment was stretched in a stepwise manner (50 µm) and the internal circumference and corresponding wall tension at each stretch were calculated and plotted to produce a resting wall tension–internal circumference curve for that particular artery using the normalization software (Powerlab, ADInstruments, Colorado Springs, CO). Uterine segments were normalized to 0.7 of L₁₃.₃ kPa, which has been found previously to be optimal for active tension development in this vessel.¹,² After obtaining the optimal tension, a 30-min equilibration period preceded the addition of test substances.

**Vascular contractile responses**

After equilibration period, endothelium-intact and -denuded arterial preparations were exposed to 80 mM KCl until reproducible and maximal depolarization-induced contractions were achieved. The presence of intact endothelium in the vascular preparations was confirmed by observing the relaxation response to 1µM acetylcholine (ACh) in rings precontracted with 1µM PE, as described previously.³,⁴ After a second round of washing and equilibration with Krebs, vascular contractile responses to KCl (80 mM) and cumulative additions of thromboxane agonist (U46619; 10⁻⁹– 3x10⁻⁶ M) phenylephrine (PE;10⁻⁹–3x10⁻⁵ M) and Ang II (10⁻¹¹ to 3x10⁻⁸M) were determined. Since tachyphylaxis develops to repeated ANG II cumulative dose response curves, only one dose response curve was obtained per tissue.¹ To assess the effects of ANG II receptors (AT₁R and AT₂R) antagonists on contractile responses to ANG II, cumulative dose response curves were constructed to ANG II in the absence of any...
ligand or after 30 min equilibration with either PD 123319 (10^{-5} M) or losartan (10^{-5} M). Again, only one dose response curve was obtained per tissue.

**Vascular relaxation responses**

Endothelium-dependent relaxation responses to cumulative concentrations of ACh (10^{-9}–10^{-5} M) and endothelium-independent relaxation responses to sodium nitroprusside (SNP, 10^{-9}–10^{-6} M) in rings precontracted with PE (1μM) were determined. Since NO, EDHF and PGI2 mediate endothelium-dependent relaxation in uterine artery, contribution of each of these endothelial factors for vasodilation was characterized by using combination of blockers.4 PGI2-mediated vascular relaxation was determined by recording ACh relaxation in the presence of inhibitors for NO (L-NAME, 10^{-4} M; Sigma) and EDHF (apamin and charybdotoxin, 10^{-7} M each; Sigma) production. NO-mediated relaxation was examined in the presence of inhibitors for PGI2 (indomethacin, 10^{-5} M; Sigma) and EDHF production. EDHF-mediated relaxation was determined in the presence of inhibitors for NO and PGI2 production. More than 1 EDHF candidate exists; however, in general, the hyperpolarizing mechanism of EDHF is considered to be mediated by SKCa and IKCa channels (blocked by apamin and charbdotoxin, respectively) on vascular endothelium.5,6

**RNA isolation and Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated from uterine arteries and placenta using TRIzol reagent (Invitrogen, Carlsbad, CA). 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 an ND-1000 Nanodrop spectrophotometer (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, Grand Island, NY). The reaction was carried out at 28°C for 15 minutes, then 42°C for 50 minutes, and stopped by heating at 94°C for 5 minutes followed by 4°C before storage at -20°C until further analysis. The mRNA levels of AT1aR, AT1bR, AT2R, eNOS, Sk3, Ik1, Cox-2, Pgis, Ip, Hif1α, Ankrd37 and Egln were quantified by using the CFX96 real-time thermal cycler (Bio-Rad). The specific TaqMan® Primers for AT1aR, AT1bR, AT2R and eNOS were ordered from Applied Biosystems (Carlsbad, CA) and the specific pairs of SYBR Green primer sequences (Table: S1) were obtained from published literature and ordered from IDT (Coralville, IA). PCR conditions for SYBR Green primers 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).PCR conditions for TaqMan® Gene Expression Assay were 2 min at 50°C and 10 min at 95°C for 1 cycle, then 15 sec at 95°C and 1 min at 60°C for 50 cycles. Results were calculated using the 2^{-ΔΔCT} method and expressed in folds increase/decrease of the gene of interest in T-treated vs control rats. All reactions were performed in duplicate, and β-actin was used as an internal control.

**Western blotting**

The uterine arteries and placenta were quickly frozen in liquid nitrogen and stored at -80°C. Arteries were homogenized in ice-cold RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium
pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin; Cell signaling Technology, Danvers, MA) containing a protease inhibitor tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail-2 and -3 (Sigma, St Louis, MO). Tissue lysates were centrifuged (14,000 g for 10 min at 4°C), and the protein content was measured by using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). The supernatant was resuspended in the NuPAGE® LDS sample buffer and reducing agent (Invitrogen). Proteins (30 μg) alongside of Precision Plus Standard (Kaleidoscope, Bio-Rad Laboratories, Hercules, CA) were resolved on 4 to 12% gradient NuPAGE® Bis-Tris Gels (Invitrogen) at 100 V for 2.0 hours at room temperature and then transferred onto Immobilon-P membranes (Millipore Inc., Billerica, MA) at 100 V for 1.5 hours. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour and then incubated overnight at 4°C with primary antibodies. The primary antibodies were eNOS (BD Transduction Labs, San Diego, CA), AT_1R, AT_2R, Hif1α, SK3, Pgis and Cox-2 (Abcam, Cambridge, MA) IP receptor (Cayman Chemical, Anna Arbor, MI), and β-actin (Cell Signaling, Danvers, MA). After washing, the membranes were incubated with secondary antibodies (anti-rabbit or -mouse conjugated with horseradish peroxidase) detected with the Chemilucent Plus Western Blot Enhancing Kit (EMD Millipore Corporation, Billerica, MA)). Densitometric measurement was done using AlphaEase FluorChem 8000 software (Alpha Innotech, Santa Clara, CA). Results were expressed as ratios of band intensity to that of β-actin.

**Data analysis**

All data are presented as mean ± SEM. Responses to contractile agents were expressed as a percentage of 80 mM K⁺ contraction. Responses to vasodilators are expressed as per cent relaxation of the initial PE contraction. Concentration–response response curves were fitted to a log-logistic sigmoid relationship, and pD₂ values (negative logarithm of the molar concentration of agonist that produced 50% of the maximal tension) and Eₘₐₓ (maximal effect) were calculated (GraphPad Prism, La Jolla, CA). Repeated measures ANOVA (treatment and time as factors) with a Bonferroni post hoc were used for comparisons of dose response curves between control and treatment groups. Per cent maximal relaxation, pD₂, and mRNA expression and protein levels were compared between control and treatment groups using unpaired Student’s t test. Statistical significance was defined as *P* < 0.05. The letter *n* represents number of rats.

**Results**

**Representative Ang II dose rose response curve**

Ang II induced dose-dependent increase in contractile response in uterine arteries of endothelium-denuded control rats. Since Ang II induces tachyphylaxis only one dose response was taken per tissue.

**T exposure does not impair vascular reactivity to K⁺ depolarization in uterine arteries.**

Vascular contractile responses to KCl (80 mM), a determination of depolarization-induced vessel contraction, was similar in endothelium-intact and –denuded arterial segments from T exposed rats compared to their respective controls (Figure S2; *n* = 8 in each group).
Losartan and PD123319 on Ang II–Induced contractions in endothelium-denuded uterine arteries

In endothelium-denuded arteries, losartan completely blocked Ang II–induced contractions in both control and T rats (Figure S3; n=5-8 in each group). On the other hand, PD123319 did not significantly affect Ang II–induced contractions in control and T rats (Figure S3; n=5-8 in each group).

T exposure does not impair endothelium-independent relaxations to sodium nitroprusside (SNP) in uterine arteries.

As shown in Figure S4, SNP-induced relaxations of the uterine arteries were not significantly different between control and T rats (n=9 in each group).

Reference List

Table S1: Primer sequences used for qRT-PCR

**TaqMan® Primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>TaqMan® Gene Expression Assay (Applied Biosystems)</th>
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<tbody>
<tr>
<td>AT1aR</td>
<td>Rn02758772_s1</td>
</tr>
<tr>
<td>AT1bR</td>
<td>Rn02132799_s1</td>
</tr>
<tr>
<td>AT2R</td>
<td>Rn00560677_s1</td>
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<td>eNOS</td>
<td>Rn02132634_s1</td>
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**SYBR Green primer sequences**

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<th>Forward 5'-3'</th>
<th>Reverse 5'-3'</th>
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<tr>
<td>Sk3</td>
<td>GATCTCTCTCTGTGGATCATTGC</td>
<td>AATCTGCTTCTCCAGGTCTTCG</td>
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<td>Ik1</td>
<td>CTACTGCACAGCAAAATCTCTCAG</td>
<td>CCTGGTATGTTGGTAGATGAGCCAC</td>
</tr>
<tr>
<td>Cox-2</td>
<td>GCAAATCCTTGCTGTCCAATC</td>
<td>GGAGAAGGCTCCAGCTTTTG</td>
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<td>Pgis</td>
<td>TCTGCAAGCTTCCAAATTTCGA</td>
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<td>Ip</td>
<td>TGGTCGAACATCCTCAGT</td>
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<td>Hif1α</td>
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<td>Ankrd37</td>
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<td>TGCCCAACAAAGACATCATC</td>
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<td>Egln1</td>
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<td>β-actin</td>
<td>CGTGAAAGATGACCCAGATC</td>
<td>CACAGCCTAGATGGCTAGTG</td>
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Table S2. Maternal, fetal and placental measures in control and T groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation length (days)</td>
<td>22±0.3</td>
<td>22±0.8</td>
</tr>
<tr>
<td>Maternal Weight* (g)</td>
<td>342.6±5.54</td>
<td>334.8±8.44</td>
</tr>
<tr>
<td>Fetal weight* (g)</td>
<td>2.4±0.29</td>
<td>1.8±0.15†</td>
</tr>
<tr>
<td>Fetal crown-rump length* (mm)</td>
<td>3.5±0.17</td>
<td>2.9±0.19†</td>
</tr>
<tr>
<td>Placental weight* (mg)</td>
<td>529±0.14</td>
<td>422±0.12†</td>
</tr>
<tr>
<td>Placental diameter* (mm)</td>
<td>1.4±0.07</td>
<td>1.3±0.09</td>
</tr>
<tr>
<td>Uterine artery diameter* (µm)</td>
<td>497±25</td>
<td>479±22</td>
</tr>
<tr>
<td>Pup birth weight (g)</td>
<td>6.01±0.18</td>
<td>5.39±0.27†</td>
</tr>
<tr>
<td>Pup crown-rump length (mm)</td>
<td>4.5±0.06</td>
<td>4.0±0.10†</td>
</tr>
<tr>
<td>Litter size</td>
<td>14±1</td>
<td>13±2</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM of 6 to 8 pregnant rats in each group. *On gestation day 20. †p<0.05 compared to control group.
**Figure S1.** Representative Ang II dose response curve

**Figure S2.** T exposure does not impair vascular reactivity to K⁺ depolarization in uterine arteries. Endothelium-intact and -denuded uterine arterial rings were isolated from control and T groups. Vascular contractile responses were taken to KCl (80 mM) and expressed as mN/mm. Values are given as means ± SEM of 8 rats in each group.
**Figure S3.** Effect of Ang II-induced contractions in presence of losartan and PD123319. Endothelium-denuded uterine arterial rings were isolated from control and T exposed groups. Vascular contractile responses were taken to cumulative additions of Ang II in absence or presence of losartan and PD123319. Values are given as means±SEM of 5-8 rats in each group.

**Figure S4.** Endothelium-independent relaxation. Uterine arterial rings were precontracted with PE (10^{-6} M) and examined for relaxation to cumulative additions of SNP. Values are given as means±SEM of 9 rats in each group.