Hypertension shows a clear age-related sex dimorphism. Nearly 1 in 3 adult Americans have hypertension. A higher percentage of men than women have hypertension until age 45 years, the percentage is similar from ages 45 to 54 years, and it becomes higher for women after that. Thus, the risk of hypertension increases in women after the onset of menopause and continues to rise with age. As a result, after puberty, only male growth-restricted offspring remain hypertensive, whereas female growth-restricted offspring stabilize their blood pressure to levels comparable to adult female controls. Because female rats reach their maximum levels of estrogen at puberty, we hypothesize that estrogen may be a factor involved in the stabilization of blood pressure in adult female growth-restricted offspring. At 10 weeks of age, female control and growth-restricted offspring underwent ovariectomy or sham surgery and insertion of a telemetry probe. Mean arterial pressure was similar at 16 weeks of age between control (123±4 mm Hg) and growth-restricted offspring (122±2 mm Hg); however, ovariectomy led to a significant increase in blood pressure in growth-restricted offspring (140±2 mm Hg; P<0.05 versus intact counterpart) with no significant effect in controls (124±1 mm Hg). Estrogen replacement by subcutaneous minipellet initiated at 14 weeks of age in a subset of ovariectomized control and growth-restricted offspring reversed the effect of ovariectomy on blood pressure in growth-restricted offspring at 16 weeks of age (111±3 mm Hg; P<0.05 versus ovariectomized counterpart); renin angiotensin system blockade also abolished ovariectomy-induced hypertension in female growth-restricted offspring (106±2 mm Hg; P<0.05 versus ovariectomized counterpart). Therefore, sex differences are observed in this model of fetal programmed hypertension, and results from this study suggest that estrogen contributes to normalization of blood pressure in adult female growth-restricted offspring. (Hypertension. 2007;50:679-685.)

Key Words: fetal programming ▪ intrauterine growth restriction ▪ ovariectomy ▪ estrogen ▪ renin angiotensin system

Alterations in the fetal environment during a critical period of fetal development result in fetal adaptive changes that lead to long-term consequences, such as increased risk for development of hypertension and cardiovascular disease later in life, an observation supported by numerous animal models. Sex differences are reported in different animal models of fetal programming; male offspring develop vascular dysfunction and hypertension, whereas female offspring seem to be protected. Therefore, a role for sex hormones is suggested in modulating cardiovascular responses to an adverse fetal environment.

In the model of fetal programming induced by placental insufficiency during late gestation in the rat, both male and female intrauterine growth-restricted (IUGR) offspring develop hypertension at prepubertal ages; however, only male IUGR offspring remain hypertensive in adulthood, whereas female IUGR offspring stabilize their blood pressure after puberty. Therefore, sex hormones may contribute to sex differences in blood pressure in adult IUGR offspring. We previously reported an important role for testosterone and the...
renal renin-angiotensin system (RAS) in the maintenance of established hypertension in adult or postpubertal male IUGR offspring.27 Based on the fact that stabilization of blood pressure in female IUGR offspring is coincident with post-puberty or the age at which female rats reach maximum levels of estrogen for this strain,28 we hypothesize that estrogen may protect against increases in blood pressure in postpubertal female IUGR offspring. In addition, based on renal RAS involvement in adult male IUGR hypertension27 and experimental studies whereby estrogen modulates the renal RAS,29–32 we hypothesize that modulation of the RAS by estrogen may contribute to blood pressure regulation in female IUGR offspring. Thus, the purpose of this study was to determine whether estrogen protects against increases in blood pressure in adult female IUGR offspring and to determine whether modulation of the RAS may serve as a mechanism by which estrogen regulates blood pressure in female IUGR offspring.

Methods

Animals
All of the experimental procedures were in accordance with National Institutes of Health guidelines with approval by the animal care and use committee at the University of Mississippi Medical Center. Rats were housed in a temperature-controlled room (23°C) with a 12:12-hour light/dark cycle, with food and water available ad libitum. Timed pregnant Sprague-Dawley rats were purchased from Harlan Inc. At day 14 of gestation, rats destined for reduced uterine perfusion were clipped as described below. All of the dams were allowed to deliver at term with offspring birth weight recorded within 12 hours of birth. At this time, the number of pups in the control and reduced uterine perfusion litter was trimmed, with a size of 8 pups per dam, to ensure equal nutrient access for all of the offspring. Animals were weighed twice weekly. Pups were weaned at 3 weeks of age. Female offspring from 13 control pregnant and 16 reduced uterine perfusion pregnancy litters were randomly assigned into 4 groups: control intact (n=14), control ovariectomized (OVX) (n=20), IUGR intact (n=14), and IUGR OVX (n=23). Implantation of telemetry probes and initiation of either OVX or sham OVX were performed at 10 weeks of age. The angiotensin-converting enzyme (ACE) inhibitor enalapril (40 mg/kg per day, PO) was administered in a randomly selected subset of intact and ovariectomized animals; control-intact+enalapril (n=7), IUGR-intact+enalapril (n=7), control-OVX+enalapril (n=7), and IUGR-OVX+enalapril (n=7). Estradiol (E2) replacement was initiated in a randomly selected subset of ovariectomized animals: control-OVX+E2 (n=6) and IUGR-OVX+E2 (n=8). Animals that did not receive enalapril or E2 were used as untreated controls for each group. Plasma for measurement of plasma renin activity (PRA) and plasma renin substrate (PRS) was collected from intact and ovariectomized animals after decapitation to prevent activation of the RAS. Serum for measurement of E2 levels was collected while animals were under anesthesia from the abdominal aorta to prevent hemolytic phenomenon in the samples. E2 levels were measured from different sets of animals at 4, 6, 8, 10, 12, and 16 weeks of age to characterize the onset of puberty and stabilization of E2 in control and IUGR offspring (n=6 to 8 per group).

Reduced Uterine Perfusion in the Pregnant Rat
Reduced uteroplacental perfusion, as described previously,22 was used for induction of IUGR. Briefly, rats undergoing surgical procedures were anesthetized with 2% isoflurane. At day 14 of gestation, a silver clip (0.203-mm ID) was placed around the lower abdominal aorta above the iliac bifurcation. Because compensation of blood flow occurs through an adaptive increase in ovarian blood flow, a silver clip was slipped around both branches of the ovarian artery (0.100-mm ID). Pregnant rats used for the control group were not exposed to surgical procedures. Based on previous observations, no differences have been noted between offspring from pregnant rats undergoing a sham operation and offspring from pregnant rats not exposed to surgical procedures (B.T.A., unpublished data, 2003).

Measurement of Mean Arterial Pressure by Radiotelemetry

Animals were anesthetized with 2% isoflurane, and a flexible catheter attached to a radio transmitter (Data Sciences) was inserted in the abdominal aorta just below the renal arteries, as described previously.27 The transmitter was secured to the abdominal muscle and remained in the abdominal cavity for the duration of the experiment. After surgery, rats were housed in individual cages positioned over an RLA-3000 radiotelemetry receiver. Rats received food and water ad libitum. Blood pressure measurements obtained during a 10-second sampling period were recorded every 10 minutes, 24 hours a day in unrestricted animals, initiated at 12 weeks of age until the end of experiments at 16 weeks of age. Mean arterial pressure (MAP) expressed in results was obtained weekly from an average of the first 3 days of the week for each rat.

Ovariectomy in Female Offspring

All of the rats undergoing surgical procedures were anesthetized with 2% isoflurane. Ovariectomy was performed at 10 weeks of age. The skin was prepared for aseptic surgery, followed by a ventral midline incision. The abdominal musculature and peritoneum were incised and the ovaries visualized. Ovarian vessels were tied off, and the ovaries were removed (OVX group). The sham operation involved a ventral midline incision. The abdominal musculature and peritoneum were incised and the ovaries visualized but not removed (intact group). The abdomen was closed in 2 layers, muscular and skin.

RAS Blockade

The ACE inhibitor enalapril (40 mg/kg per day, PO) was administered in the drinking water from 14 weeks of age until the end of the experiment at 16 weeks of age.

E2 Replacement

17β-E2 valerate minipelleils (1.5 mg for 21-day release) were used for continuous release of hormone (Innovative Research of America) at a dose chosen to mimic the normal E2 levels observed in adult Sprague-Dawley female control animals (25±2 ng/dL). This dose characterized the average E2 level of synchronized adult female control and IUGR rats housed in the same cage and represented a combination of the different stages of the estrous cycle as confirmed by cytology characteristic of vaginal smearing.

Measurement of E2 Levels

Serum E2 levels were determined with a commercially available radioimmunoassay kit (Ultra-Sensitive E2 RIA DSL-4800).

Measurement of PRA

PRA was measured by radioimmunoassay using a modification of the method by Haber et al,33 with angiotensin I (AI) standards, tracer, and antibody from National Bureau of Standards, New England Nuclear, and Arnel, respectively.

Measurement of PRS

PRS was measured by radioimmunoassay, as described previously.34

Isolation of Total Kidney RNA and Real-Time PCR

Total RNA was used for quantification of the mRNA by Real-Time PCR. Kidneys were removed, quick frozen in liquid nitrogen, and stored at −80°C. Each kidney was first ground using a liquid nitrogen–chilled mortar and pestle, and total RNA was isolated using a guanidine thiocyanate, acid phenol/chloroform procedure (TALLY RNA kit, Ambion). All of the RNA isolates were treated
with DNase (DNA-free kit, Ambion) to remove DNA. Total RNA (2 μg) was reverse transcribed using a modified Moloney murine leukemia virus-derived reverse transcriptase and a unique blend of oligo(dT) and random hexamer primers (iScript™cDNA Synthesis kit, Bio-Rad). The resulting cDNA (1 μL) was amplified by real-time PCR using SYBR Green (iQ™ SYBR Green Supermix, Bio-Rad) as fluorophore in an iCycler real-time thermal cycler (Bio-Rad). ACE and ACE2 mRNA expressions were assessed using the RT2 PCR Primer Set for Rat ACE and ACE2 (SuperArray Bioscience Corporation). Results were calculated using the 2^−ΔΔCt method and were expressed in folds increase/decrease of the gene of interest in IUGR versus control rats. All of the reactions were performed in triplicate, and β-actin was used as an internal control (RT-PCR Primer and Control Set, Invitrogen).

Statistics
GB-STAT version 7 for MS Windows was used for all of the statistical analyses. For comparison made between groups, ANOVA, with adjustments for multiple comparisons, was used. For 2-group comparisons, t test was used. A value of P < 0.05 was considered statistically significant.

Results

Body Weight
Weight at birth was significantly reduced in female IUGR offspring from reduced uterine perfusion dams as compared with female control offspring from control dams (Table). At 16 weeks of age, body weight did not differ in the comparison of female IUGR to female control offspring (Table). Therefore, female IUGR offspring exhibited catch-up growth as differences in body weight were normalized by 16 weeks of age. Body weight did not differ on comparison in any group (Table). Thus, neither ovariectomy nor ACE inhibition affected body weight in control or IUGR offspring by 16 weeks of age.

MAP
As reported previously by our group and now confirmed by telemetry, MAP did not differ in adult female IUGR offspring as compared with adult female control offspring (Figure 1). Ovariectomy induced hypertension in adult IUGR-OVX offspring (mean increase of 18 mm Hg; Figure 1) yet had no significant effect on blood pressure in adult control-OVX offspring (mean increase: 1 mm Hg). E2 replacement for 2 weeks initiated at 14 weeks of age in postpubertal offspring significantly reduced blood pressure by 16 weeks of age in control-OVX offspring (mean decrease: 20 mm Hg; P < 0.05 versus control-OVX offspring) and IUGR-OVX offspring (mean decrease: 29 mm Hg; P < 0.05 versus IUGR-OVX offspring; Figure 2). However, E2 replacement abolished the significant difference in MAP observed in the comparison of control-OVX and IUGR-OVX offspring normalizing blood pressure in IUGR-OVX offspring to values comparable to control-OVX offspring.

The ACE inhibitor enalapril initiated at 14 weeks of age decreased blood pressure in IUGR-OVX and control-OVX rats (Figure 3). However, the depressor response to ACE inhibition was greater in IUGR-OVX rats (mean decrease: 35 mm Hg versus untreated IUGR-OVX rats; P < 0.01) as compared with control-OVX rats (mean decrease: 5 mm Hg versus untreated control-OVX rats; P < 0.05; Figure 3). ACE inhibition led to a significant decrease in blood pressure in intact-IUGR (mean decrease: 9 mm Hg) but not intact-control offspring (mean decrease: 3 mm Hg) as compared with their intact untreated counterparts. MAP did not significantly differ on comparison of treated intact-control and treated OVX-control offspring. In addition, ACE inhibition abolished the significant difference in MAP observed on comparison of untreated OVX-IUGR and untreated OVX-control offspring.

Plasma E2 Levels
Serum E2 levels did not differ in the comparison of adult female control and adult female IUGR offspring at 16 weeks of age (Figure 4a). E2 levels were measured at 4, 6, 8, 10, 12, and 16 weeks of age in a separate set of animals. E2 levels oscillated from 4 to 6 weeks of age between 0.7 and 0.9 ng/dL, and no significant differences were observed in the comparison of IUGR to control offspring at these ages.

Table. Body Weight of Control and IUGR Offspring

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Birth, Body Weight, g</th>
<th>16 wk of Age, Body Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact UT</td>
<td>Intact Enalapril</td>
</tr>
<tr>
<td>Control</td>
<td>5.9 ± 0.1 (n = 34)</td>
<td>297 ± 5 (n = 7)</td>
</tr>
<tr>
<td>IUGR</td>
<td>4.9 ± 0.1 (n = 37)†</td>
<td>315 ± 7 (n = 7)</td>
</tr>
</tbody>
</table>

*P < 0.05 vs control offspring.

Weight at birth was measured within 12 hours. UT indicates untreated animals; Enalapril, animals treated with an ACE inhibitor; OVX, animals subjected to ovariectomy; intact, animals subjected to sham OVX. All of the data are expressed as mean ± SEM. t test was used for comparison at birth and multiple comparisons ANOVA for multiple comparisons at 16 weeks of age.

### Figure 1
Ovariectomy and blood pressure in IUGR offspring. MAP was measured by radiotelemetry from 12 to 16 weeks of age in animals that underwent either sham (intact) or OVX at 10 weeks of age. Control intact (n = 7), control OVX (n = 7), IUGR intact (n = 7), and IUGR OVX (n = 8). *P < 0.01 vs IUGR intact. All data are expressed as mean ± SEM.
However, E2 levels were increased at 8 weeks of age, a value that correlates with puberty for this rat strain, and were maintained at this level up to 16 weeks of age or the end of the study, with no significant differences observed in the comparison of female IUGR with control offspring (Figure 4a). At puberty, recognized by vaginal opening, daily vaginal smearing was performed to identify the stage of estrous cycle at the time of E2 measurement. Although E2 levels may represent a combination of the different stages of the estrous cycle, biweekly E2 levels reflected synchronized cycles in female rats housed in the same cage. E2 levels were significantly decreased at 6 weeks after ovariectomy in both control and IUGR offspring in comparison with their intact counterparts (Figure 4b). E2 replacement reinstated E2 levels to levels comparable with intact animals (Figure 4b).

PRA and PRS
No significant differences in PRA or PRS were observed in the comparison of control and IUGR rats at 16 weeks of age (PRA: 4 ± 1 versus 5 ± 1 nmol of AI per liter per hour; PRS: 25 ± 2 versus 27 ± 6 nmol of AI per liter per hour; control versus IUGR, respectively). Ovariectomy had no effect on either PRA or PRS levels in control or IUGR rats (PRA: 4 ± 1 versus 3 ± 1 nmol of AI per liter per hour; PRS: 27 ± 1 versus 23 ± 2 nmol of AI per liter per hour; control versus IUGR, respectively).

Renal ACE and ACE2 mRNA Expression
Renal ACE2 mRNA expression was significantly increased in adult female intact IUGR offspring in comparison with adult female control offspring (Figure 5). Ovariectomy had no effect on renal ACE mRNA expression in either control or IUGR offspring. However, renal ACE2 mRNA expression was significantly decreased by ovariectomy in IUGR-OVX rats (Figure 5); ovariectomy had no effect on renal ACE2 mRNA expression in control-OVX rats.

Discussion
Hypertension induced by placental insufficiency exhibits sex-specific differences in adult IUGR offspring. Hypertension in female IUGR offspring from reduced uterine perfusion dams returns to normotensive values in adulthood, whereas male IUGR offspring remain hypertensive. We reported recently that testosterone contributes to the maintenance of established hypertension in postpubertal male IUGR offspring. This study demonstrates that estrogen protects against increases in blood pressure in postpubertal female IUGR offspring. Importantly, this study also demonstrates a potential role for the RAS as an underlying mechanism in mediating hypertension induced by ovariectomy in adult female IUGR offspring.

Normalization of blood pressure in postpubertal female IUGR offspring occurred in conjunction with attainment of adult female E2 levels for this strain. Furthermore, ovariectomy led to the development of significant elevations in MAP in adult female IUGR offspring with no effect in adult female control offspring. Thus, the possibility that E2 plays a protective role in the normalization of arterial pressure was strongly suggested. However, no differences in E2 levels were observed in the comparison of adult female control and
We reported previously that ACE inhibition abolishes hypertension in adult male IUGR offspring. To clarify the importance of E2 on blood pressure control in female IUGR offspring, E2 replacement in ovariectomized rats was initiated to return estrogen levels to those observed in adult female rats of this strain.28 Estrogen replacement abolished hypertension induced by ovariectomy in adult female IUGR offspring and normalized blood pressure to levels observed in the ovariectomized control plus estrogen replacement group, suggesting that estrogen does provide a protective status in adult female IUGR offspring. However, the protective role of E2 may not be directly related to the level of E2, per se, but to the effect of E2 on other systems controlling blood pressure in adult female IUGR offspring.

Epidemiological studies of hormonal replacement therapy are controversial as to whether E2 provides a protective status in postmenopausal women.35-39 However, E2 is associated with protective cardiorenal effects in many animal models of hypertension.11,12,40,41 and the deleterious effects of ovariectomy, such as induced hypertension, renal injury, or endothelial dysfunction, are reversed with E2 therapy.12,13,41 The cardiorenal protective effect of E2 seems to be complex and includes a wide range of regulatory system involvement with a role for the RAS strongly suggested in both human and animal studies.30,42-46

We reported previously that ACE inhibition abolishes hypertension in adult male IUGR offspring.27 Furthermore, activation of the renal RAS but not peripheral RAS is associated with hypertension in adult male IUGR rats,47 suggesting a role for the renal RAS in adult male IUGR hypertension. To investigate whether the RAS contributes to hypertension induced by ovariectomy in adult female IUGR offspring, we examined the effect of RAS blockade using the ACE inhibitor enalapril. Enalapril abolished hypertension induced by ovariectomy in adult female IUGR offspring, suggesting that the RAS plays a critical role in mediating hypertension induced by ovariectomy in female IUGR offspring. Thus, ovariectomy leads to development of hypertension in adult female IUGR offspring, an effect reversed by RAS blockade.

One potential mechanism for the protective status mediated by E2 in adult IUGR female offspring may involve modulation of the renal RAS by estrogen. ACE- and ACE2-dependent pathways generate peptides, angiotensin II, and angiotensin-1-7, respectively, which are critical for blood pressure regulation.48,49 Angiotensin-1-7 acts as a negative regulator of the vasoconstrictor effects of angiotensin II, thus suggesting that ACE2 provides a counterregulatory balance to ACE.13,50,51 ACE, ACE2, and the angiotensin type 1 receptor are components of the RAS associated with the cardiorenal protective effects of E2.31,52-55 Modulation of the RAS by E2 involves alterations in the vasoconstrictor-vasodilator actions of the RAS by influencing the ACE and ACE2 pathways.31,32,51,53-56 Various animal models have been used to investigate participation of RAS in the regulatory effects of E2.32,46,52,56-58 Although the exact mechanism(s) remains unclear, it is suggested that when ACE activity is greater than ACE2, vasoconstriction because of angiotensin II will predominate, and hypertension will be induced.46 Because estrogen is shown to regulate the renal ACE message in the rat,30 we determined whether renal ACE and ACE2 message expression were altered in IUGR offspring relative to control offspring and whether renal ACE and ACE2 message expressions were altered in response to ovariectomy. Significant elevations in renal ACE mRNA expression were observed in intact adult female IUGR offspring.
spring in comparison with intact adult female control offspring. Ovariectomy induced a significant decrease in renal mRNA expression of ACE2 in adult female IUGR offspring with no effect in adult female controls. Ovariectomy had no effect on renal ACE mRNA expression in adult female IUGR offspring. Thus, loss of ovarian function may decrease the vasoconstrictor effect provided by the ACE2 pathway leading to increases in blood pressure in postpubertal female IUGR offspring. Ovariectomy did not affect blood pressure or renal ACE and ACE2 mRNA expression in control animals. Therefore, the abnormal response to loss of ovarian function on blood pressure regulation in adult female IUGR offspring may reflect permanent alterations in the regulatory systems important in the long-term control of arterial pressure regulation that develop as a consequence of fetal adapted changes to placental insufficiency.

Hypertension is present in prepubertal female IUGR offspring; however, estrogen protects against hypertension in postpubertal female IUGR offspring. Modulation of the RAS, in particular, the ACE2 pathway, by estrogen may be one mechanism critical to the normalization of blood pressure in adult female IUGR offspring. However, other factors may be modulated by estrogen, and mechanism(s) mediating ovariectomy-induced elevations in arterial pressure in adult female IUGR offspring may also involve oxidative stress. Investigation into the regulation of these factors by estrogen in this model of IUGR will be a very exciting opportunity for future investigations.

Perspectives
The influence of the fetal environment is a novel factor in the etiology of hypertension. Various animal models of programmed hypertension have been used to study the mechanisms underlying the pathophysiology of this condition and the regulatory systems involved in these mechanisms. Placental insufficiency results in IUGR offspring that reveal sex-specific differences in the development of programmed hypertension. Adult female IUGR offspring seem protected while the ovaries remain intact, and the RAS with participation of the ACE2 counterbalance pathway may be involved. Thus, mechanisms common to sex differences in this model of programmed hypertension may enlighten the complex mechanisms underlying the sex differences in human hypertension.

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References
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