Intrauterine Growth Restriction and Androgen Receptor

Chronic Blockade of the Androgen Receptor Abolishes Age-Dependent Increases in Blood Pressure in Female Growth-Restricted Rats


Abstract—Intrauterine growth restriction induced via placental insufficiency programs a significant increase in blood pressure at 12 months of age in female growth-restricted rats that is associated with early cessation of estrous cyclicity, indicative of premature reproductive senescence. In addition, female growth-restricted rats at 12 months of age exhibit a significant increase in circulating testosterone with no change in circulating estradiol. Testosterone is positively associated with blood pressure after menopause in women. Thus, we tested the hypothesis that androgen receptor blockade would abolish the significant increase in blood pressure that develops with age in female growth-restricted rats. Mean arterial pressure was measured in animals pretreated with and without the androgen receptor antagonist, flutamide (8 mg/kg/day, SC for 2 weeks). Flutamide abolished the significant increase in blood pressure in growth-restricted rats relative to control at 12 months of age. To examine the mechanism(s) by which androgens contribute to increased blood pressure in growth-restricted rats, blood pressure was assessed in rats untreated or treated with enalapril (250 mg/L for 2 weeks). Enalapril eliminated the increase in blood pressure in growth-restricted relative to vehicle- and flutamide-treated controls. Furthermore, the increase in medullary angiotensin type 1 receptor mRNA expression was abolished in flutamide-treated growth-restricted relative to untreated counterparts and controls; cortical angiotensin-converting enzyme mRNA expression was reduced in flutamide-treated growth-restricted versus untreated counterparts. Thus, these data indicate that androgens, via activation of the renin–angiotensin system, are important mediators of increased blood pressure that develops by 12 months of age in female growth-restricted rats. (Hypertension. 2016;67:1281-1290. DOI: 10.1161/HYPERTENSIONAHA.116.07548.)

Key Words: blood pressure • low birth weight • renin-angiotensin system • testosterone • women’s health

Men have higher blood pressure compared with age-matched women in young adulthood; however, this sex difference is lost with the onset of menopause when the risk for cardiovascular disease in women surpasses their age-matched male counterparts.1 The prevalence of hypertension is greater in postmenopausal women relative to men of similar age,2,3 and the age-related decline in blood pressure control is greater in women relative to men,4 despite women being more compliant with their therapeutic regimen.5 Yet, the mechanisms responsible for the decrease in blood pressure control in women after menopause are unknown.

Numerous physiological systems are implicated in the pathogenesis of postmenopausal hypertension, including the renin–angiotensin system (RAS),6 the endothelin system,7 and alterations in sex steroids.8 Circulating testosterone levels are elevated in postmenopausal women compared with premenopausal women,5 and testosterone is positively associated with blood pressure after menopause.10 Thus, these studies suggest that androgens play a contributory role in the development of hypertension in women as they age and pass through the menopausal transition. However, the exact mechanism(s) by which a hyperandrogenic state results in an increase in blood pressure in women is not well-defined.

The experimental model of the aged female spontaneously hypertensive rat (SHR) mimics many of the characteristics reported for women with postmenopausal hypertension, including cessation of cycling, age-dependent increases in blood pressure, and significant increases in circulating testosterone levels compared with young female SHRs.11 The mechanism responsible for the increase in blood pressure in the aged female SHR is multifactorial and involves the RAS,12 endothelin system,13 and eicosanoids.14 However, in these studies, monotherapy to block each of these individual pathways fails to normalize blood pressure in the aged female.
SRH relative to young female SHR counterparts; an observation also noted with triple therapy. Testosterone is implicated in the activation of each of these pathways. Therefore, these findings suggest that blockade of the androgen receptor might be a potential therapeutic option for postmenopausal hypertension.

Numerous epidemiological studies indicate that low birth weight individuals exposed to intrauterine growth restriction (IUGR) are at an increased risk for cardiovascular disease and high blood pressure in later life. In addition to the increased risk for cardiovascular disease, low birth weight women exhibit an increased prevalence for early age at menopause compared with normal birth weight counterparts. To investigate the long-term effects of IUGR on later cardiovascular risk, our laboratory uses an experimental model of IUGR induced by placental insufficiency at day 14 of gestation in the Sprague-Dawley rat. Female growth-restricted rats are normotensive in young adulthood; however, they develop a significant age-related increase in blood pressure by 12 months of age. In addition, female growth-restricted rats enter reproductive senescence at an earlier age compared with their female normal birth weight counterparts, control offspring of sham-operated dams. Circulating testosterone levels are significantly elevated in female growth-restricted rats at 12 months of age that have ceased cycling, whereas serum estradiol levels do not differ relative to age-matched female controls. Because an elevation in testosterone is observed in female growth-restricted rats at 12 months of age in association with a significant elevation in blood pressure, the aim of this study was to test the hypothesis that chronic blockade of the androgen receptor would abolish the significant age-related increase in blood pressure in female growth-restricted offspring. Based on studies suggesting that testosterone is elevated in postmenopausal women compared with premenopausal women and that testosterone may function as an activator of the RAS in postmenopausal women, we also tested the hypothesis that involvement of testosterone in the pathogenesis of age-related increases in blood pressure in female growth-restricted rats at 12 months of age incorporates androgen receptor–dependent activation of the RAS.

Materials and Methods

All experimental procedures were conducted in accordance with National Institutes of Health guidelines with all protocols approved by the Animal Care and Use Committee at the University of Mississippi Medical Center. In brief, female IUGR offspring exposed to reduced uterine perfusion at day 14 of gestation or female control offspring from sham-operated dams were divided into 2 groups: daily injections of androgen receptor antagonist, flutamide (8 mg/kg SC, n=9/10; Sigma Aldrich, Saint Louis, MO) or daily injections of vehicle (ethanol in castor oil, n=11/12) for 2 weeks. Mean arterial pressure was measured in conscious, chronically instrumented rats followed by collection of plasma and kidneys for further analysis. In a separate cohort of animals, a subgroup of female offspring were pretreated with angiotensin-converting enzyme (ACE) inhibitor, enalapril (250 mg/L), in the drinking water for 2 weeks (n=6–7) followed by measurement of mean arterial pressure (MAP). A more detailed Methods section is available in the online-only Data Supplement.

Statistics

Data are presented as mean values±SE, with n representing the number of female offspring with different mothers per group. MAP, serum testosterone, and estradiol levels within the different study groups were compared using 2-way repeated-measures analysis of variance (Prism 5.0, GraphPad, San Diego). Post hoc testing was performed using Bonferroni’s post hoc test to use multiple comparisons where appropriate. Differences were reported as significant when P<0.05.

Results

Birth Weight and Body Weight in Female Control and Growth-Restricted Rats at 12 Months of Age

Birth weight was significantly reduced in growth-restricted females compared with same-sex controls (5.70±0.07 versus 4.87±0.09 g; P<0.05, control versus IUGR, respectively). Body weight did not differ between vehicle-treated control and vehicle-treated growth-restricted offspring at 12 months of age (288±11 versus 267±9 g; P>0.05, control versus IUGR, respectively). Flutamide treatment had no effect on body weight in control or growth-restricted offspring (269±3 versus 263±6 g; P>0.05, control versus IUGR, respectively) at 12 months of age.

Mean Arterial Pressure and Sex Steroids in Female Control and Growth-Restricted Rats at 12 Months of Age: The Effect of Blockade of the Androgen Receptor

MAP was significantly elevated in vehicle-treated growth-restricted offspring compared with control counterparts at 12 months of age (P<0.05; Figure 1A). Chronic blockade of the androgen receptor with flutamide abolished the increase in MAP in flutamide-treated growth-restricted relative to flutamide-treated control offspring at 12 months of age (P>0.05; Figure 1A). Circulating testosterone levels were significantly elevated in vehicle-treated growth-restricted offspring compared with controls (P<0.05; Figure 1B). Chronic androgen receptor blockade significantly reduced circulating testosterone levels in female growth-restricted offspring relative to vehicle-treated growth-restricted offspring (P<0.05, flutamide-treated IUGR versus vehicle-treated IUGR, respectively; Figure 1B). Estradiol levels were comparable between vehicle-treated controls and growth-restricted offspring (P>0.05), and flutamide treatment had no effect on estradiol levels in either group (Figure 1C). As observed previously, female growth-restricted offspring were predominantly in persistent estrous at 12 months of age, whereas age-matched control offspring retained a normal pattern of estrous cyclicity. At 18 months of age, chronic flutamide did not significantly reduce blood pressure in control or growth-restricted offspring at 18 months of age (Figure S1 in the online-only Data Supplement).

Intrarenal mRNA Expression of the Renin–Angiotensin System in Female Control and Growth-Restricted Rats at 12 Months of Age

Renin mRNA expression did not differ on comparison of vehicle-treated control and growth-restricted offspring in the cortex or medulla at 12 months of age. However, renin mRNA expression was significantly increased 3-fold in cortex and 2-fold in medulla of flutamide-treated growth-restricted offspring compared with their flutamide-treated control counterpart and 2-fold relative to their
vehicle-treated growth-restricted counterpart (Figure 2A and 2B). Cortical and medullary angiotensinogen mRNA expression was comparable between vehicle-treated control and growth-restricted offspring but renal cortical angiotensinogen mRNA expression was significantly elevated 2-fold in flutamide-treated growth-restricted compared with vehicle-treated growth-restricted and flutamide-treated controls; an observation not observed within the renal medulla (Figure 2C and 2D). Renal cortical and medullary ACE mRNA did not differ between vehicle-treated control and growth-restricted offspring (Figure 2E and 2F). Yet, there was a significant 2-fold reduction in renal cortical ACE mRNA expression in flutamide-treated growth-restricted compared with vehicle-treated growth-restricted and flutamide-treated controls. The reduction in ACE mRNA expression within the medulla was abolished by flutamide treatment (Figure 2G). Chronic blockade of the androgen receptor also did not alter medullary ACE mRNA expression in control offspring but cortical ACE mRNA expression was significantly decreased 2-fold in flutamide-treated growth-restricted offspring relative to vehicle-treated counterparts (Figure 2G and 2H). There was no difference in cortical or medullary ACE2 mRNA expression between vehicle-treated control and growth-restricted offspring (Figure 2G and 2H); however, medullary expression of ACE2 mRNA was significantly increased 3-fold in flutamide-treated control offspring compared with vehicle-treated control and 2-fold relative to flutamide-treated growth-restricted offspring (Figure 2H). Flutamide-treated growth-restricted offspring did not exhibit a significant change in ACE2 mRNA expression compared with vehicle-treated groups.

**Mean Arterial Pressure and Plasma Renin Activity in Female Control and Growth-Restricted Rats at 12 Months of Age: The Effect of Blockade of the Renin Angiotensin System**

MAP was significantly elevated in vehicle-treated growth-restricted offspring relative to control counterparts at 12 months of age (P<0.05, IUGR versus control; Figure 3A). Blockade of the RAS with the ACE inhibitor, enalapril, abolished the significant increase in MAP in growth-restricted relative to vehicle-treated growth-restricted offspring (P<0.05, IUGR versus control; Figure 3A). Plasma renin activity did not differ between vehicle-treated control and growth-restricted offspring (P>0.05; Figure 3B). After chronic blockade of the RAS with enalapril, plasma renin activity was significantly elevated in control and growth-restricted offspring to comparable levels (P<0.05, vehicle-treated versus enalapril-treated counterparts; Figure 3B).

**Intrarenal Androgen and Angiotensin Receptor mRNA Expression in Female Control and Growth-Restricted Rats at 12 Months of Age**

Cortical angiotensin receptor (ATR) 1a expression did not differ on comparison of control and growth-restricted offspring (Figure 4A and 2G); however, medullary expression of ATR1 a mRNA was significantly increased 3-fold in flutamide-treated control offspring compared with vehicle-treated control and 2-fold relative to flutamide-treated growth-restricted offspring (Figure 2H). Flutamide-treated growth-restricted offspring did not exhibit a significant change in ATR1a mRNA expression compared with vehicle-treated groups. Chapter 2H. Flutamide-treated growth-restricted offspring did not exhibit a significant change in ATR1a mRNA expression compared with vehicle-treated groups.
Figure 2. mRNA levels of kidney renin–angiotensin system (RAS) components from vehicle-treated and flutamide-treated female control and intrauterine growth-restricted (IUGR) rats by real-time reverse transcriptase polymerase chain reaction (RT-PCR) at 12 months of age. Cortical renin mRNA expression (A), medullary renin mRNA expression (B), cortical angiotensinogen mRNA expression (C), medullary angiotensinogen mRNA expression (D), cortical angiotensin-converting enzyme (ACE) mRNA expression (E), medullary ACE mRNA expression (F), cortical ACE2 mRNA expression (G), medullary ACE2 mRNA expression (H) in female vehicle-treated control (n=8) and IUGR offspring (n=7) and flutamide-treated control (n=9) and IUGR offspring (n=8). Data are expressed as fold changes relative to the mean expression level of the vehicle-treated control rats that were arbitrarily defined as 1. *P<0.05 versus vehicle-treated IUGR. †P<0.05 versus flutamide-treated control. ‡P<0.05 versus vehicle-treated control.
treatment did not alter expression (Figure 4C). Medullary ATR1b expression was significantly increased 2-fold in growth-restricted offspring compared with controls; chronic flutamide abolished this increase (Figure 4D). Androgen receptor mRNA expression in both the cortical and medullary regions did not differ on comparison of vehicle-treated control and growth-restricted offspring; intrarenal androgen receptor mRNA expression was not altered by treatment with flutamide (Figure 4E and 4F).

**Discussion**

Previously, we reported that female growth-restricted rats are normotensive in young adulthood relative to age-matched female controls. However, a more recent study from our laboratory demonstrates that female growth-restricted rats exhibit a significant increase in blood pressure relative to age-matched female controls at 12 months of age that is associated with cessation of estrous cyclicity and a significant increase in circulating levels of testosterone. The main finding from this study indicated that the increase in blood pressure in female growth-restricted offspring at 12 months of age relative to age-matched controls was abolished by chronic treatment with the androgen receptor antagonist, flutamide, with no effect on blood pressure in age-matched female controls. Furthermore, flutamide had no effect on blood pressure in female control offspring at 18 months of age that were in persistent estrous. Therefore, these results suggest that the reduction in blood pressure in response to chronic blockade of the androgen receptor is specific to female rats that exhibit early cessation of estrous cyclicity after fetal exposure to IUGR, not reproductive senescence per se. This study also determined the importance of the RAS in the pathogenesis of androgen-dependent increased blood pressure in female growth-restricted rats at 2 months of age. Intrarenal expression of components of the RAS, including renin, angiotensinogen, and ACE, were not significantly increased in vehicle-treated female growth-restricted offspring compared with vehicle-treated controls at 12 months of age. Yet, cortical ACE mRNA expression was significantly reduced in flutamide-treated growth-restricted rats in conjunction with a significant increase in renin and angiotensinogen mRNA expression at 12 months of age, perhaps indicative of feedback from the reduction in renal cortical ACE expression. Medullary ATR1a and AT1b mRNA expression were increased in female growth-restricted offspring relative to control at 12 months of age; this increase was not present in rats treated with flutamide, implicating modulation of the intrarenal RAS by the androgen receptor in female growth-restricted rats. The importance of the RAS in the pathogenesis of increased blood pressure in female growth-restricted offspring at 12 months of age was determined by the effect of RAS blockade. Blockade of the RAS using the ACE inhibitor, enalapril, completely abolished the increase in blood pressure in enalapril-treated growth-restricted offspring relative to enalapril- and vehicle-treated controls. Therefore, these results indicate that the RAS is also a contributory factor in the pathogenesis of increased blood pressure at 12 months of age in female growth-restricted offspring.

Numerous experimental models of developmental programming induced by exposure to a mild developmental insult, such as moderate maternal protein restriction (9% versus 18% protein), fetal exposure to increased glucocorticoids, or placental insufficiency, report a sex difference in blood pressure, with male offspring in young adulthood exhibiting a higher blood pressure relative to age-matched female counterparts, whereas female offspring are normotensive in young adulthood relative to their control counterparts. However, female rat offspring exposed to a moderate (9% versus 18% protein) reduction in maternal protein intake during gestation or placental insufficiency induced via a reduction in uterine perfusion during fetal life develop a significant increase in blood pressure by 1 to 2 years of age relative to their control counterparts, suggesting that females do not remain protected against increased cardiovascular risk. The pathogenesis for the age-related increase in blood pressure in female offspring in models of developmental insult that mimic the pathophysiological causes for low birth weight is not clear. Hypertension in male growth-restricted offspring relative to male control in young adulthood is abolished.

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**Figure 3.** The effect of renin–angiotensin system (RAS) blockade with the angiotensin-converting enzyme (ACE) inhibitor, enalapril (250 mg/L for 2 weeks), on mean arterial pressure (MAP; A) and plasma renin activity (PRA; B) measured in female control and intrauterine growth-restricted (IUGR) rats at 12 months of age. MAP was measured at 12 months of age in chronically instrumented, conscious animals via carotid catheter. PRA was measured using radioimmunoassay. Vehicle-treated control n=7, vehicle-treated IUGR n=6, enalapril-treated control n=6, enalapril-treated IUGR n=6. *P<0.05 versus vehicle-treated control. †P<0.05 versus vehicle-treated IUGR.
by castration, suggesting that hypertension in male rats is testosterone-dependent. Ovariectomy induces a significant increase in blood pressure in female growth-restricted offspring in young adulthood, which is abolished by estrogen replacement, implicating that estrogen may be protective. Therefore, previous studies from our laboratory indicate that sex steroids contribute to the sexual dimorphism of blood pressure in growth-restricted offspring in young adulthood. Although testosterone levels are increased in female growth-restricted offspring at 12 months of age, circulating estradiol levels do not differ relative to age-matched female control.

Female growth-restricted rats at 12 months of age exhibit persistent estrous compared with age-matched female controls, which retain a normal pattern of estrous cyclicity. A reduction in ovarian follicle number and early disruption in estrous cyclicity associated with an increase in circulating levels of testosterone is reported in female rats exposed to a moderate reduction (9% versus 18% protein) in maternal protein intake. Earlier age at menopause is reported in low birth weight women and in women exposed in utero to famine. Therefore, these studies suggest that insults during early life program early reproductive senescence.

Figure 4. Angiotensin receptor (ATR) and androgen receptor mRNA expression in the kidney from vehicle-treated and flutamide-treated female control and intrauterine growth-restricted (IUGR) rats at 12 months of age by real-time reverse transcriptase polymerase chain reaction (RT-PCR). Cortical ATR1α mRNA expression (A), medullary ATR1α mRNA expression (B), cortical ATR1β mRNA expression (C), medullary ATR1β mRNA expression (D), cortical androgen receptor mRNA expression (E), medullary androgen receptor mRNA expression (F) in female vehicle-treated control (n=8) and IUGR offspring (n=7) and flutamide-treated control (n=9) and IUGR offspring (n=8). Data are expressed as fold changes relative to the mean expression level of the vehicle-treated control rats that were arbitrarily defined as 1.

*P<0.05 versus vehicle-treated control. †P<0.05 versus vehicle-treated IUGR.
It is well established that early-onset menopause increases the risk for cardiovascular disease.\(^{40}\) Although the mechanisms involved are not yet known, testosterone is indicated as a potential mediator.\(^{41}\) Testosterone is positively associated with blood pressure in women across their lifespan,\(^{42,43}\) with the association significant in postmenopausal women\(^{42,43}\) and in women with polycystic ovary syndrome,\(^{44,45}\) a syndrome often characterized by a state of hyperandrogenemia. Although Huang et al. report that administration of exogenous testosterone in women with endogenous low testosterone levels does not alter blood pressure,\(^{45}\) administration of exogenous testosterone\(^{46}\) or \(5\alpha\)-dihydrotestosterone\(^{47}\) increases blood pressure in female rats in experimental models of increased blood pressure. The detrimental effect of exogenous testosterone on cardiovascular health in women is not well-defined\(^{48}\) but the use of exogenous testosterone for treatment of numerous disorders in women is currently contraindicated.\(^{49}\)

The effect of androgen deprivation therapy on cardiovascular health in women is also inconclusive.\(^{50}\) Experimental studies indicate that blockade of androgen receptor is beneficial against increases in blood pressure in the female rat. The transgenic TGR(mREN2)27 rat overexpresses the mouse renin transgene and spontaneously develops hypertension in association with inappropriate activation of the rat RAS.\(^{51}\) Endogenous testosterone levels are not elevated in the female TGR(mREN2)27 rat. Yet, blockade of the androgen receptor with flutamide abolishes hypertension in conjunction with a decrease in plasma renin expression and plasma renin activity in the absence of an effect on testosterone.\(^{52}\) Thus, these findings suggest that endogenous testosterone is the mediator of inappropriate activation of the RAS in the female TGR(mREN2)27 rat. Treatment with flutamide also reduces blood pressure in female rats exposed to a severe (5\% versus 18\%) reduction in maternal protein intake during gestation.\(^{53}\) Unlike more physiologically relevant models of developmental insult, blood pressure is significantly elevated as early as 3 months of age in female offspring exposed to a severe maternal protein restriction during fetal life.\(^{53}\) Like the female TGR(mREN2)27 rat, circulating levels of testosterone are not elevated in female rats in this model, indicating an androgen independent effect of flutamide on blood pressure.\(^{52}\) The mechanism by which flutamide reduces blood pressure in female protein-restricted rats was not investigated; however, estrogen levels are decreased and flutamide reverses this effect.\(^{53}\) Sex steroids mediate their actions via genomic and non-genomic pathways. Circulating levels of testosterone and estradiol were not altered by blockade of the androgen receptor in our study, suggesting a lack of a nongenomic vascular effect on blood pressure. Thus, we tested the hypothesis that blockade of the androgen receptor modulated expression of the intrarenal RAS to reduce blood pressure in female growth-restricted rats at 12 months of age.

Postmenopausal women have higher risk for cardiovascular risk compared with premenopausal women.\(^{54}\) The exact mechanisms responsible for postmenopausal hypertension are unknown, but evidence shows it is a multifactorial disease.\(^{55}\) Blood pressure increases with age after estrous cycling ceases in the female SHR,\(^{11}\) suggesting that aged female SHRs may be a model for the study of postmenopausal hypertension. Serum testosterone levels are increased in aged female SHRs relative to young same-sex counterparts.\(^{11}\) Plasma renin activity is also elevated relative to the young female SHR, implicating a role for the RAS.\(^{12}\) In the present study, blockade of the androgen receptor abolished the increase in blood pressure in female growth-restricted rats relative to control at 12 and 18 months of age, with no effect on blood pressure in control offspring at either age. Only female control rats at 12 months of age exhibited different phases of the estrous cycle. Thus, the decrease in blood pressure by flutamide was specific to rats exposed to IUGR and not related to the presence of persistent estrous. ACE inhibition also abolished the increase in blood pressure in female growth-restricted rats at 1 year of age, suggesting an important role for the RAS. Intrarenal ACE mRNA expression did not differ in vehicle-treated female control in this study relative to vehicle-treated female growth-restricted offspring. Yet, intrarenal ACE mRNA expression was decreased in flutamide-treated growth-restricted rats relative to flutamide-treated control, suggesting that the effect of flutamide on expression of renal ACE was specific to the female growth-restricted rat. Renal ACE activity was not examined. However, exogenous estradiol can modulate ACE activity in the female Sprague–Dawley rat by reducing ACE mRNA.\(^{56}\) Intrarenal mRNA expression of renin and angiotensinogen mRNA expression did not differ in vehicle-treated rats but were significantly increased in flutamide-treated growth-restricted offspring relative to untreated counterparts. The increase in expression of these factors may involve feedback from inhibition of ACE by the actions of flutamide. ACE2 mRNA expression was increased in flutamide-treated female control relative to untreated control, and ACE2 was the only component of the RAS altered in female control offspring by flutamide. A direct test of flutamide on renal ACE2 mRNA expression or activity is not yet been reported. However, Gupte et al. report that renal ACE2 mRNA expression is increased in male C57Bl/6 mice relative to females, suggesting a correlative role.\(^{57}\) Estrogen increases adipocyte ACE2 mRNA expression in female C57Bl/6 mice,\(^{58}\) and we previously demonstrated that ovariectomy reduces ACE2 mRNA expression and activity in female growth-restricted offspring in young adulthood.\(^{59}\) Thus, the mechanism by which ACE2 was increased by chronic blockade of the androgen receptor in the absence of a change in estradiol is not clear. The increase in renal AT1R expression was abolished by flutamide in correlation with a loss of increased blood pressure in female growth-restricted offspring relative to control. Expression of the AT1R is androgen-dependent,\(^{60}\) suggesting that increased expression of the AT1R in female growth-restricted offspring is androgen-dependent. Therefore, our study indicated a direct role for the RAS in the pathogenesis of increased blood pressure that develops in female growth-restricted rats that exhibit early reproductive senescence at 12 months of age. Yet, diuretics are the most common treatment for hypertension in postmenopausal women, whereas men are reported to respond better to ACE inhibitors.\(^{59}\) ACE
inhibitors are also the most prescribed antihypertensive medication for low birth weight Medicaid male recipients, whereas low birth weight is associated with a greater use of calcium channel blockers in low birth weight women.60

Experimental models of hypertension exhibit a sex difference in the blood pressure response to different antihypertensive therapies.61–65 Angiotensin receptor blocker production increases a greater decrease in blood pressure in aged male SHR compared with aged female SHR,65 despite no sex difference in intrarenal mRNA expression of ACE or the AT1R. Oxidative stress may play a more important role in blood pressure control in male rats in some experimental models of hypertension relative to their female counterparts.61,64 Furthermore, male rats in models of developmental insult exhibit a blood pressure response to blockade of the endothelin type A receptor that is absent in female litters.62,63 Therefore, understanding the pathogenesis of hypertension after reproductive senescence is needed to prescribe the appropriate therapeutic approach for postmenopausal women. Future studies will address the specificity of targets. Inhibition of blood pressure in female growth-restricted offspring. Understanding the pathogenesis of hypertension that develops after a developmental insult in women as they age and are also at risk for earlier age of reproductive senescence is also necessitated.

Clinical Perspectives

Low birth weight women are at an increased risk for cardiovascular disease and earlier age for onset of menopause compared with normal birth weight counterparts; yet, less is known about the pathogenesis of hypertension in individuals, in particular women, exposed to a prenatal insult. This study demonstrates an important role for androgens in the development of age-related increases in blood pressure in female growth-restricted rats and also implicates the RAS as a contributor to the developmental programming of increased blood pressure that develops with age in the female growth-restricted rat.

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Disclosures

None.

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**Novelty and Significance**

**What Is New?**

- Our study demonstrates that testosterone acting through the androgen receptor contributes to the age-related increase in blood pressure in female growth-restricted rats. Our study further reveals that the renin-angiotensin system is also a contributor to the development of increased blood pressure in female growth-restricted rat at 12 months of age via the actions of the androgen receptor.

**What Is Relevant?**

- The risk for hypertension in women greatly increases after the onset of menopause, yet the pathogenesis of postmenopausal hypertension is still unclear. The pathogenesis of increased blood pressure in women born low birth weight is also unknown, and studies looking at the effect of age, and the pathophysiological mechanisms that contribute to a greater prevalence of hypertension in low birth weight women as they age, are limited. Findings from our study that indicate a role for androgens in the pathogenesis of increased blood pressure that develops with age in female growth-restricted rats should encourage further examination into the relevance of testosterone and its detrimental effect on blood pressure in women, in particular in low birth weight women in later life.

**Summary**

Further studies are necessary to discern the impact of age and reproductive status on effectiveness of an antihypertensive regimen in low birth weight women.
Chronic Blockade of the Androgen Receptor Abolishes Age-Dependent Increases in Blood Pressure in Female Growth-Restricted Rats

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Supplemental Materials

Chronic Blockade of the Androgen Receptor Abolishes Age-Dependent Increases in Blood Pressure in Female Growth-Restricted Rats.


Running Title: Female, IUGR, Testosterone and Blood Pressure
MATERIALS AND METHODS

Animals.
All experimental procedures were conducted in accordance with National Institutes of Health guidelines. All protocols were approved 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–h light-dark cycle with food and water available ad libitum. Timed pregnant Sprague Dawley rats were purchased from Harlan Inc (Indianapolis, IN). At day 14 of gestation, rats intended for reduced uterine perfusion were clipped as described or a sham procedure was conducted to generate control offspring. All pregnant rats were allowed to deliver at term with birth weights recorded within 12 h of delivery. Pups in control and reduced uterine perfusion litters were culled to four female and four male pups per dam to allow equal nutrition access for all offspring; however, only female offspring were utilized in this study. Only one pup per litter was used for each study parameter.

Reduced Uterine Perfusion in the Pregnant Rat.
Reduced utero-placental perfusion or the sham procedure was performed on day 14 of gestation as previously described to induce intrauterine growth restriction (IUGR) (1).

Chronic blockade of the androgen receptor.
Female offspring were divided into 2 groups at one year of age. Group 1 consisted of female control and growth-restricted rats administered vehicle (ethanol in castor oil, n=11/12). Group 2 consisted of female control and growth-restricted rats receiving the androgen receptor antagonist, flutamide (8 mg/kg SC, n=9/10) (Sigma Aldrich, Saint Louis, Missouri, USA). Treatment was administered via subcutaneous injection from 50 to 52 weeks of age at a dose previously shown to reduce blood pressure in the rat (2). In a smaller cohort, this protocol was performed in animals at 18 months of age.

Chronic blockade of the renin angiotensin system.
Female offspring were treated with enalapril, an angiotensin converting enzyme (ACE) inhibitor, for 2 weeks (250g/L) in the drinking water (3). Vehicle treated animals were maintained on drinking water ad libitum.

Measurement of mean arterial pressure.
Mean arterial pressure (MAP) was measured in control and growth-restricted male rats as previously described (4). Briefly, under isoflurane anesthesia, rats were surgically implanted with flexible catheters (PE-50) in the right carotid artery for measurement of MAP then externalized through the nape of the neck. Animals were allowed to recover for 24 hours. MAP was measured in conscious, chronically instrumented rats using a data acquisition kit (DATAQ Instruments, Akron, OH) via connecting carotid catheter to a pressure transducer with a computer continuously recording pressures. At the end of the experiment, animals were euthanized to collect blood and tissue samples for further analysis.

Isolation and quantitation of mRNA using real-time PCR (qRT-PCR).
Kidneys were flash frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from the renal cortex and medulla using NucleoSpin RNA (Machery-Nagel Inc., Bethelhem, PA). cDNA was synthesized from 1µg of RNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). qRT-PCR was performed using iQ SYBR Green Supermix (BioRad, Hercules, CA) and the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA). The following primer sequences (Integrated DNA Technologies, USA) were used for quantitation of renin (forward: tgatcctggtcatgtctactcc, reverse: cctcttgtagcttcagtctcc), angiotensinogen (forward: caggtcaggatgcagaagatg, reverse: ggatagctgtgcttgtctgg), ACE (forward: caccaattcctccatgttcac,
reverse: tgtagagaagccaaacctgga), angiotensin converting enzyme 2 (ACE2) (forward: gataacaatgccacactacc, reverse: gtctgaatgataacagctgga), angiotensin receptor type 1a (ATR1a) (forward: cactatcgcatactgcacc, reverse: ctctcagctctgcacattc), angiotensin receptor type 1b (ATR1b) (forward: tgtctctgacactattaattgc, reverse: gcaacacagccttcca) and androgen receptor (AR) (forward: gtgggaagtaatagtcgatggg, reverse: agtgaaatgggaccttggatg). Levels of mRNA expression were calculated using the mathematical formula for delta/delta CT recommended by Applied Biosystems (Applied Biosystems User Bulletin, No. 2, 1997, Foster City, CA).

**Serum Testosterone and Estradiol Levels.**
Serum estradiol levels were measured using a commercially available kit as previously described (3). Serum testosterone levels were measured using a commercially available kit (IBL America, Minneapolis, MN).

**Plasma Renin Activity.**
PRA was measured by radioimmunoassay using a modification of the method by Haber et al. (5) with angiotensin I (AI) standards, tracer, and antibody from National Bureau of Standards, New England Nuclear, and Arnel, respectively.

**Estrous cycle.** The phase of estrous cycle was determined by vaginal cytology at the same time in the morning using a sterile cotton-tipped swab moistened with sterile saline to swipe the dorsal vaginal wall then immediately rolled on a glass slide. After drying, the slides were stained with methylene blue to determine the following features: cornified epithelial cells, nucleated epithelial cells, leukocytes, and mucus. The stage of estrous cycle was determined via the following criteria 1) metestrus was identified as predominantly leukocytes and cornified epithelial cells 2) diestrus was identified as predominantly leukocytes, some nucleated epithelial cells, and mucus 3) proestrus was identified as predominantly nucleated epithelial cells, few cornified epithelial cells, and few leukocytes; and 4) estrus was identified as predominantly cornified epithelial cells.

**Statistics.**
Data are presented as mean values ± SE, with n representing the number of female offspring with different mothers per group. MAP, serum testosterone and estradiol levels within the different study groups were compared using two-way repeated-measures ANOVA (Prism 5.0, GraphPad, San Diego, CA). Post hoc testing was performed using Bonferroni’s post hoc test to utilize multiple comparisons where appropriate. Differences were reported as significant when P < 0.05.
References


Figure S1. Effect of androgen receptor antagonism on mean arterial pressure (MAP) in female intrauterine growth-restricted (IUGR) rats at 18 months of age. Parameters were measured at 18 months of age in chronically instrumented, conscious animals pretreated with the androgen receptor antagonist, flutamide (8 mg/kg/day for 2 weeks). Data values represent mean±SEM.