Original Article

Pregnancy Protects Hyperandrogenemic Female Rats From Postmenopausal Hypertension

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Abstract—Polycystic ovary syndrome, the most common endocrine disorder in women of reproductive age, is characterized by hyperandrogenemia, obesity, insulin resistance, and elevated blood pressure. However, few studies have focused on the consequences of pregnancy on postmenopausal cardiovascular disease and hypertension in polycystic ovary syndrome women. In hyperandrogenemic female (HAF) rats, the hypothesis was tested that previous pregnancy protects against age-related hypertension. Rats were implanted with dihydrotestosterone (7.5 mg/90 days, beginning at 4 weeks and continued throughout life) or placebo pellets (controls), became pregnant at 10 to 15 weeks, and pups were weaned at postnatal day 21. Dams and virgins were then aged to 10 months (still estrous cycling) or 16 months (postcycling). Although numbers of offspring per litter were similar for HAF and control dams, birth weights were lower in HAF offspring. At 10 months of age, there were no differences in blood pressure, proteinuria, nitrate/nitrite excretion, or body composition in previously pregnant HAF versus virgin HAF. However, by 16 months of age, despite no differences in dihydrotestosterone, fat mass/or lean mass/body weight, previously pregnant HAF had significantly lower blood pressure and proteinuria, higher nitrate/nitrite excretion, with increased intrarenal mRNA expression of endothelin B receptor and eNOS (endothelial nitric oxide synthase), and decreased ACE (angiotensin-converting enzyme), AT1aR (angiotensin 1a receptor), and endothelin A receptor than virgin HAF. Thus, pregnancy protects HAF rats against age-related hypertension, and the mechanism(s) may be due to differential regulation of the nitric oxide, endothelin, and renin-angiotensin systems. These data suggest that polycystic ovary syndrome women who have experienced uncomplicated pregnancy may be protected from postmenopausal hypertension. (***Hypertension***. **2020;75:00-00. DOI: 10.1161/HYPERTENSIONAHA.120.15504.)**

● **Data Supplement**

Key Words: aging ■ endothelin ■ menopause ■ nitric oxide ■ renin-angiotensin system

Polycystic ovary syndrome (PCOS) is the most common endocrine pathology in women of reproductive age, affecting 5% to 10% of the population, often beginning in adolescence.[1–3] PCOS in young women is characterized by hyperandrogenemia, modest increases in blood pressure, insulin resistance, and increased inflammation.[2–4] With aging, hyperandrogenemia does not abate, even following menopause.[5–7] The clinical guidelines for PCOS diagnosis have only been in place since 2002 to 2004[1]; thus there are few studies that have focused on the consequences of aging (ie, post-menopause) on cardiovascular disease (CVD) risk in PCOS women, due to the lack of aging populations in which PCOS had been definitively diagnosed, that is, with androgen measurements. Furthermore, to our knowledge, there are no studies in which the effect of prior pregnancy on postmenopausal CVD and hypertension in PCOS women has been studied.

PCOS women may have difficulty becoming pregnant and have a higher incidence of requiring assisted reproduction, such as in vitro fertilization.[8,9] Persson et al[8] reported that Scandinavian PCOS women take longer to become pregnant and have fewer children than women without PCOS, but once pregnant, the probability of childbirth was similar in PCOS women versus controls. Hu et al[10] reported that PCOS women had elevated blood pressure during pregnancy, as measured by ambulatory recording, but there were no data on prepregnancy blood pressures, nor did the blood pressures reach guideline levels required for treatment of hypertension.

One may surmise that exposure to cardiovascular and metabolic risk factors throughout the entirety of their reproductive lives would predispose PCOS women to early CVD risk. However, this supposition is controversial.[11–14] For example, recent reviews question whether women with PCOS have greater morbidity and mortality than the general population of postmenopausal women, and point out that data are scarce as to the postmenopausal health of PCOS women, and even suggest that PCOS women are not different from the general

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population or actually may be protected from CVD.[11,15–17] One study showed that PCOS women go through menopause 4 years later than age-matched controls,[18] which may imply protection from CVD due to longer exposure to estradiol.

In recent years, we have studied the hyperandrogenemic female (HAF) rat that mimics many of the characteristics of women with PCOS.[19] Female Sprague-Dawley rats are given dihydrotestosterone, beginning shortly after weaning and continued throughout their lives[19]; dihydrotestosterone is used since it cannot be converted to estradiol. The serum levels of androgens are similar to levels found in PCOS women and do not affect endogenous synthesis of testosterone and estradiol.[19] By 14 to 16 weeks of age, HAF rats develop obesity, hyperlipidemia, insulin resistance, inflammation, and elevated blood pressure.[19] We found that the elevated blood pressure in the young HAF rat is mediated in part by increased intrarenal vascular 20-HETE,[20] and increased sympathetic activation.[21] We have also characterized the HAF rats as they stop estrous cycling, by 12 to 13 months of age.[22] Blood pressure continues to increase with aging in HAF rats, and they become hypertensive.[22]

Thus using the HAF rat model in the current study, we tested the hypothesis that pregnancy would protect against the hypertension that occurs in virgin HAF rats with aging (16 months of age) and evaluated the intrarenal mRNA expression of genes known to contribute to postmenopausal hypertension, such as the nitric oxide (NO) pathway, the renin-angiotensin system, and the endothelin system. We also determined if differences in blood pressure occurred at 10 months of age before cessation of estrous cycling, and whether there were differences in body composition that occurred during pregnancy or with aging that could impact blood pressure in HAF rats.

Materials and Methods

The data from this work are available from the corresponding author upon reasonable request.

Animal Model

Female Sprague-Dawley rats were obtained at 3 weeks of age from the vendor (Envigo, Indianapolis, IN) and allowed to equilibrate in a temperature-controlled environment with 12-hour:12-hour light:dark cycle for 1 week. As shown in Figure S1A and S1B in the Data Supplement, rats were randomly selected to be implanted with either 5α-dihydrotestosterone or placebo pellets to generate HAF or controls, respectively, as we previously described.[19,22] All protocols followed the ARRIVE Guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and complied with the Guidelines for the Care and Use of Laboratory Animals by the National Institutes of Health.

Estrous Cycle and Pregnancy

HAF rats exhibited a 6-day estrous cycle rather than the typical 4-day cycle as found in controls.[19,22] In separate groups of rats and as shown in Figure S1A and S1B, at 2 months of age, body composition (lean and fat mass) was measured in HAF and control rats (n=4–14/group) by echo-magnetic resonance imaging[23] (Methods in the Data Supplement) and then at 2.5 to 3 months of age, HAF and control rats were paired with Sprague-Dawley males. Pregnancies occurred in ≈60% of HAF rats and 99% of control rats. When pregnancy was detected, as denoted by increased body weight, males were removed from the cage. HAF and control dams were allowed to deliver, and within 12 hours of delivery, the number of pups in each litter (both control and HAF) were counted (n=16–25 litters/group), body weights were obtained, and anogenital distances were measured to differentiate males from females (≈3–4 mm in males and ≈1–2 mm in females). Pups were reweighed at 48 hours. The weights of male and female pups were averaged per litter at both time points and were then averaged as groups: male control, male HAF, female control, and female HAF pups. All dams were allowed to suckle their pups, and pups were weaned at 21 days of age.

In a separate group of rats, body composition was measured before pregnancy in HAF rats and in age-matched controls (noted above), as shown in Figure S1A and S1B, and body composition was measured again by echo-magnetic resonance imaging 2 days postpartum in HAF and control rats and compared to age-matched virgin controls and HAF rats.

Aging Protocols

As shown in Figure S1A and S1B, following weaning of the pups, control and HAF previously pregnant rats were allowed to age to 10 or 16 months (n=4–16/group). Age-matched HAF and control virgins were also allowed to age to 10 or 16 months. At 10 and 16 months of age, 24-hour urine collections were made for measurement of protein and nitrate/nitrite excretion in virgin and previously pregnant HAF rats, as we previously described[23,24] (Methods in the Data Supplement). Body composition and oral glucose tolerance were also measured at 10 and 16 months of age in HAF virgins and previously pregnant rats, as previously described.[23] Mean arterial pressure (MAP), systolic, and diastolic blood pressure were measured by radiotelemetry for 4 days at 10 and 16 months of age in both virgin and previously pregnant HAF and control rats, as previously described (Methods in the Data Supplement).

Upon completion of MAP in 16 months old virgin and previously pregnant HAF rats (n=6/group), blood samples were taken for plasma dihydrotestosterone and insulin that were measured as we previously described[23] (Methods in the Data Supplement). In additional group of rats, kidneys from virgin and previously pregnant control and HAF rats removed and flash-frozen in liquid nitrogen for real-time quantitative polymerase chain reaction measurement of mRNA.

Gene Expression

To determine mRNA expression of genes in systems that are known to play a role in blood pressure control, quantitative polymerase chain reaction was performed in kidneys from virgin and previously pregnant control and HAF rats (n=6–12/group), as we previously described[23] (Methods in the Data Supplement). The data for eNOS (endothelial nitric oxide synthase), renin, angiotensinogen, ACE (angiotensin-converting enzyme), AT1aR (angiotensin 1a receptor), pre-pro-ET-1 (endothelin), ET_A receptor (R), and ET_BR (endothelin B receptor) were factored for the geometric mean of four housekeeping genes (shown in Figure S2).

Statistical Analyses

All data are expressed as means±SEM. Two-way ANOVA was used to determine the differences among groups in most studies. For the data in Tables 1 and 2, 2-way ANOVA with repeated measures was used. The Student *t* test was used to determine differences in number of pups per litter, plasma dihydrotestosterone, and insulin levels, body weight gains prepregnancy and postpregnancy. Uncorrected Fisher LSD test was used for post hoc tests when necessary. Values of $P \leq 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc, V6.0c, San Diego, CA).

Results

Characteristics of the Offspring of HAF and Control Pregnancies

As shown in Figure 1A, there were no differences in the number of offspring per litter in HAF and control pregnancies (control: 12±1; HAF: 11±1 pups/litter). Birth weights for both male and female HAF offspring were less than male or female offspring born to control dams (Figure 1B), and HAF offspring weights remained lower at 48 hours postnatally

Table 1. BW and Body Composition in Pregnant Control and HAF Rats Before Pregnancy and 48 Hours After Delivery

Parameter		Pregnant Control (n=5)	Pregnant HAF (n=3)	Interaction	Time	Androgens
BW, g	Prepregnancy	197.8±5.2	251.0±3.0*	*P*=0.53	*P*<0.01	*P*<0.01
	Postdelivery	263.6±12.3†	323.7±7.1*			
BW gain during pregnancy, g		65.8±7.4	72.7±4.3			
Fat mass, g	Prepregnancy	16.5±0.6	18.5±1.0	*P*=0.86	*P*=0.04	*P*=0.41
	Postdelivery	21.7±2.5	22.9±1.6			
Fat mass (% of BW)	Prepregnancy	8.4±0.4	7.4±0.4	*P*=0.97	*P*=0.67	*P*=0.07
	Postdelivery	8.2±0.6	7.1±0.4			
Lean mass, g	Prepregnancy	174.9±6.1	227.4±2.5*	*P*=0.54	*P*<0.01	*P*<0.01
	Postdelivery	232.2±10.1‡	288.9±6.6*‡			
Lean mass (% of BW)	Prepregnancy	88.4±0.8	90.6±0.1*	*P*=0.45	*P*=0.30	*P*=0.02
	Postdelivery	88.1±0.4	89.3±0.2			

Values represent mean±SEM. Fat and lean masses were determined by Echo-MRI as described in Methods. Statistical analyses by ANOVA with repeated measures and uncorrected Fisher LSD; significance was defined as $P \leq 0.05$. BW gain was compared by *t* test. BW indicates body weight; HAF, hyperandrogenemic female; and MRI, magnetic resonance imaging.

*$P<0.05$, compared with pregnant controls, $P \leq 0.05$.

†$P<0.05$ compared with virgin HAF.

‡$P<0.05$, compared with prepregnancy of the same group.

(Figure 1C). Female HAF offspring also weighed less than male HAF offspring at both ages (Figure 1B). These data support significant impacts of both androgens and sex on the differences, but no interaction was noted.

Mean Arterial Pressure

As shown in Figure 2A, at 16 months of age, MAP in virgin HAF rats was higher than in other groups, and previous pregnancy attenuated the hypertension in HAF rats. MAP in previously pregnant HAF rats was slightly higher than in previously pregnant control rats. Thus, there were significant impacts of both androgens and parity on MAP at 16 months, but no interactions noted.

To determine whether there were differences in MAP before cessation of estrous cycling, MAP was measured in virgin and previously pregnant control and HAF rats, 10 months of age. As shown in Figure 2B, only androgens, not parity, impacted the hypertension in HAF versus control rats, and there was no interaction noted.

When blood pressures were compared at 10 and 16 months of age in HAF rats (Figure 2C), MAP was similar between virgin HAF at 10 months of age, previously pregnant HAF at 10 months of age, and previously pregnant HAF at 16 months of age. Importantly, MAP increased significantly in virgin HAF rats between 10 and 16 months of age. There was significant interaction between age and parity in these studies.

Interestingly, as shown in Figure 2D and 2E, changes in systolic blood pressure rather than diastolic blood pressure

Table 2. BW and Body Composition in Pregnant and Virgin HAF Before Pregnancy and 48 Hours After Delivery

Parameter		Age-Matched Virgin HAF (n=4)	Pregnant HAF (n=3)	Interaction	Time	Pregnancy
BW, g	Prepregnancy	243.0±13.2	251.0±3.0	*P*=0.30	*P*<0.01	*P*=0.91
	Postdelivery	327.3±7.1*	323.7±7.1*			
BW gain during pregnancy, g		84.3±7.9	72.7±4.3			
Fat mass, g	Prepregnancy	18.0±1.6	18.5±1.0	*P*=0.052	*P*<0.01	*P*=0.18
	Postdelivery	32.8±3.8*	22.9±1.6*,†			
Fat mass (% of BW)	Prepregnancy	7.4±0.6	7.4±0.4	*P*=0.047	*P*=0.09	*P*=0.09
	Postdelivery	10.0±0.8*	7.1±0.4†			
Lean mass, g	Prepregnancy	219.7±2.5	227.4±2.5	*P*=0.92	*P*<0.01	*P*=0.71
	Postdelivery	282.1±17.8*	288.9±6.6*			
Lean mass (% of BW)	Prepregnancy	90.4±0.8	90.6±0.1	*P*<0.01	*P*<0.01	*P*=0.12
	Postdelivery	86.1±0.8*	89.3±0.8*,†			

Values represent mean±SEM. Fat and lean masses were determined by Echo-MRI as described in Methods. Statistical analyses by ANOVA with repeated measures and uncorrected Fisher LSD; significance was defined as $P \leq 0.05$. BW gain was compared by *t* test. BW indicates body weight; HAF, hyperandrogenemic female; and MRI, magnetic resonance imaging.

*$P<0.05$, compared with prepregnancy of the same group.

†$P<0.05$ compared with virgin HAF.

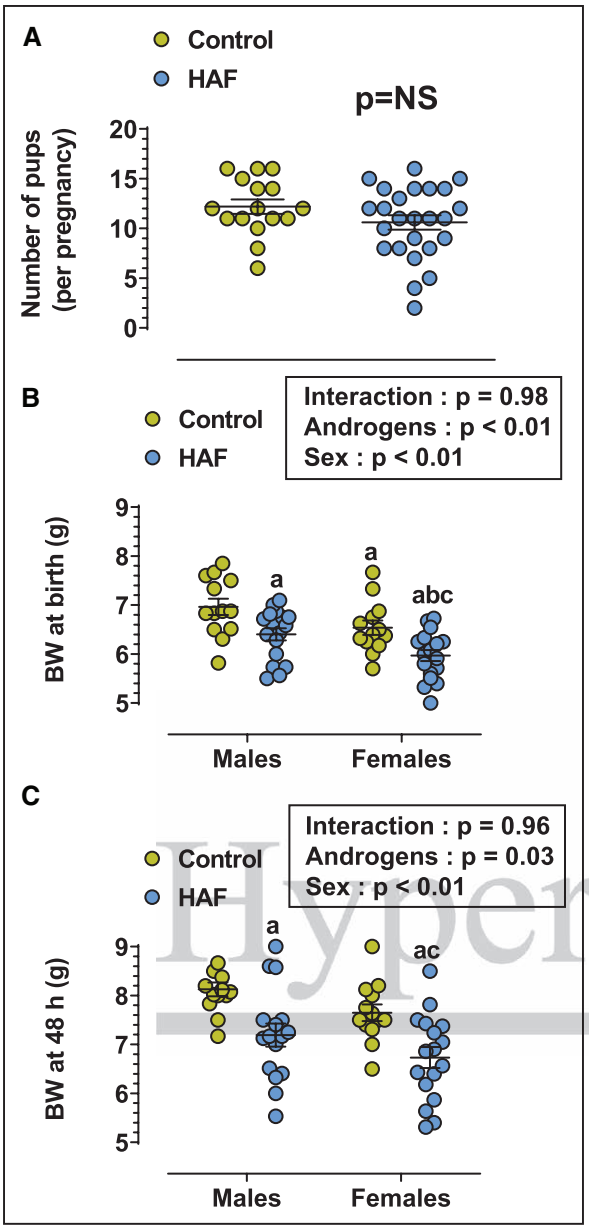

Figure 1. Number of pups per litter and body weights of hyperandrogenemic female (HAF) and control offspring. **A**, There were no differences in average numbers of pups per litter between control and HAF dams (*P*=NS). **B**, Birth weights were lower in male and female HAF offspring than control offspring, and female HAF birth weights were lower than male HAF. **C**, At postnatal day 2, body weights remained lower in male and female HAF offspring than controls. Statistical significance using the Student *t* test (**A**) or 2-way ANOVA (**B** and **C**) was defined as $P \leq 0.05$. **A**, $P \leq 0.05$ compared with control males; **B**, $P \leq 0.05$ compared with HAF male offspring; **C**, $P \leq 0.05$ compared with control female offspring. BW indicates body weight; and ns, nonsignificant.

accounted for the increased blood pressure in virgin HAF at 16 months of age.

Potential Mechanisms That Could Contribute to Lower Blood Pressure in Previously Pregnant HAF Rats, Aged 16 Months

Dihydrotestosterone Levels

We have shown previously that dihydrotestosterone levels are increased ≈3-fold with dihydrotestosterone treatment compared with placebo controls.[19,20,25,26] To determine if there were differences in dihydrotestosterone levels in control and previously pregnant HAF rats that could account for the differences in MAP at 16 months of age, plasma dihydrotestosterone was measured. However, dihydrotestosterone was not different between virgin and previously pregnant HAF rats (virgin HAF: 102.8±22.9; previously pregnant HAF [n=6]: 101.9±22.9 pg/mL; p=NS).

Body Composition, Prepregnancy, and Postdelivery

To determine whether pregnancy itself affected body composition in HAF rats that could impact later in life blood pressure, body weight, fat and lean masses, and their ratios to body weight were measured prepregnancy or 48 hours postdelivery in control or HAF rats and compared with age-matched virgin HAF rats. As shown in Table 1, before and after pregnancy, HAF rats had higher body weight than controls. Body weight increased similarly with time in both pregnant groups. Time and androgens provided the major differences between the groups, with no interactions noted. Changes in fat mass were affected only by time, not androgens. Fat mass factored for body weight was not affected by pregnancy or time. Lean mass was higher in HAF rats than control before and after pregnancy, and both time and androgens were the major factors for the differences, with no interactions noted. When factor for body mass, only androgens were the main factor for differences between control and HAF pregnant rats with no interactions found. As shown in Table 2 comparing pregnant and virgin HAF rats, before pregnancy there were no differences in the rats. With time, both groups increased body weights to similar extent, and there was no difference in body weight gain. Fat mass did not increase to the same extent in pregnant HAF rats as in controls, but there was no interaction noted. When fat mass was factored for body weight there was interaction noted, however. Lean mass increased in both groups over the time of pregnancy with no interactions. However, when factored for body weight, there was interaction with time with pregnant groups increasing lean mass more than control virgin HAF.

Body Composition and Metabolic Factors With Aging

To determine if there were differences in body composition with aging in HAF rats that may contribute to the differences in MAP, fat, and lean masses were measured. As shown in Table 3, both age and pregnancy status significantly affected body weight with no significant interaction, with body weight being similar in virgin and previously pregnant HAF at 10 months but was reduced in previously pregnant HAF at 16 months compared with virgins. Fat mass was higher in 16 months old groups, whereas lean mass was not different among the groups. Lean mass was not different among the groups, but lean mass/body weight ratios were significantly lower in HAF at 16 months than the other groups. Fat mass, fat mass/body weight ratios, and lean mass/body weight ratios were only affected by time, not parity, with no significant interactions.

To determine if there were differences in insulin resistance between virgin and previously pregnant HAF rats, plasma insulin levels were measured, and oral glucose tolerance test was

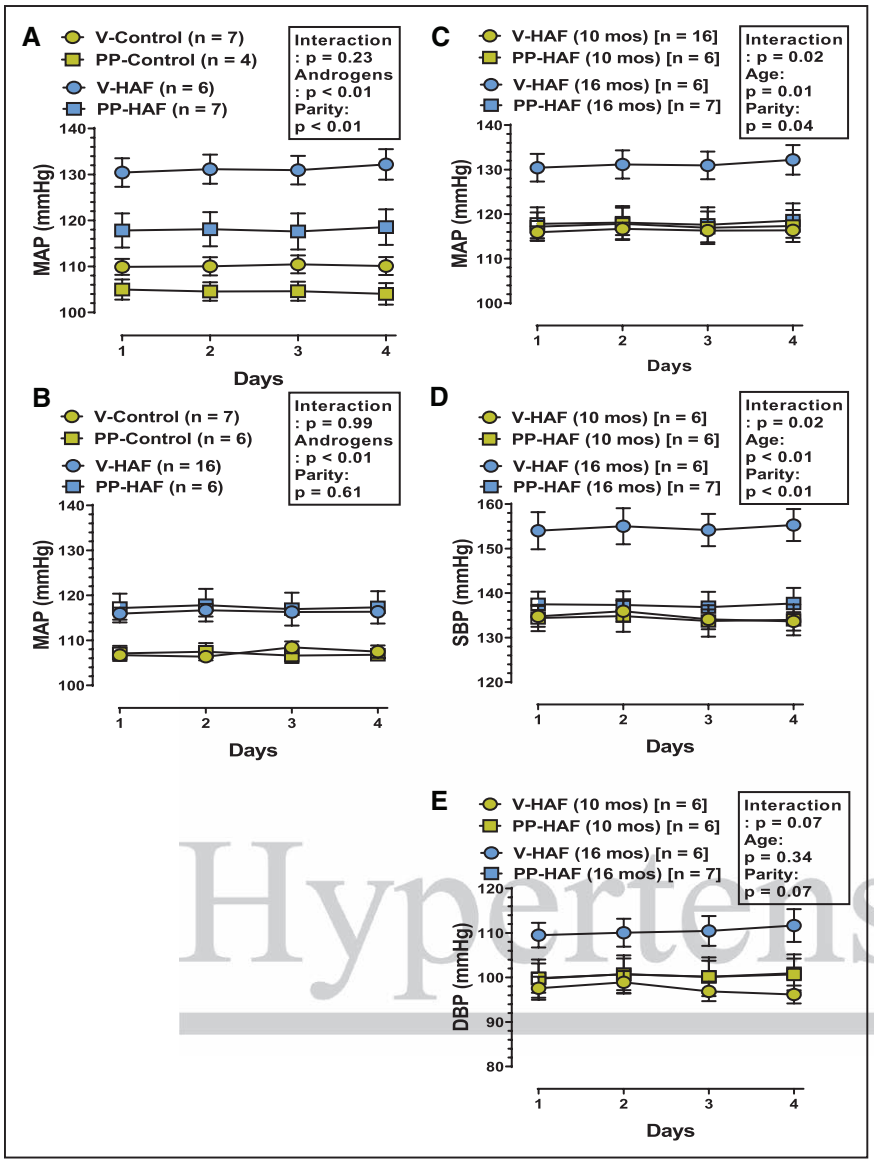

Figure 2. Blood pressure in virgin and previously pregnant hyperandrogenemic female (HAF) and control rats at 10 or 16 mo of age. Statistical significance was determined by 2-way ANOVA with repeated measures and defined as $P \leq 0.05$. **A**, At 16 mo of age, mean arterial pressure (MAP) was similar in previously pregnant and virgin controls. MAP in previously pregnant HAF rats was not different than virgin controls but was higher than previously pregnant controls. MAP was higher in virgin HAF rats than all controls or previously pregnant HAF rats. **B**, MAP at 10 mo of age was higher in virgin and previously pregnant HAF rats than in virgin or previously pregnant controls. Pregnancy had no effect on MAP in either controls or HAF rats. **C**, Comparison of MAP at 10 and 16 mo of age in virgin and previously pregnant HAF rats. MAP was not different in previously pregnant HAF rats at 16 mo of age compared with either virgins or previously pregnant HAF rats at 10 mo of age. However, MAP increased in virgin HAF rats between 10 and 16 mo of age. **D**, Comparison of systolic blood pressure (SBP) at 10 and 16 mo of age in virgin and previously pregnant HAF rats. SBP was not different in previously pregnant HAF rats at 16 mo of age compared with either virgins or previously pregnant HAF rats at 10 mo of age. However, SBP increased in virgin HAF rats between 10 and 16 mo of age, and there was significant interaction with age and parity. **E**, Comparison of diastolic blood pressure (DBP) at 10 mo and 16 mo of age in virgin and previously pregnant HAF rats. DBP was not different in vigin or previously pregnant control rats at 10 or 16 mo of age. There was no effect of age or parity and there were no interactions.

performed. Plasma insulin was not different between the HAF groups at 16 months of age (virgin HAF [n=14]: 1.3±0.2; previously pregnant HAF [n=10]: 1.1±0.2 ng/mL; *P*=NS). Oral glucose tolerance, as shown in Figure S3, was not affected by previous pregnancy in HAF rats at either 10 or 16 months of age. Fasting blood glucose level was also not elevated in either HAF groups at either age, showing rats were not diabetic.

Proteinuria and Nitrate/Nitrite Excretion

We determined if proteinuria increased with aging in HAF rats and whether pregnancy affected the levels. As shown in Figure 3A, both aging and parity impacted the level of proteinuria with no interactions noted. Proteinuria was higher in 16-month-old HAF virgins than virgin or previously pregnant HAF at 10 months, and previous pregnancy attenuated proteinuria in HAF rats, 16 months of age.

As shown in Figure 3B, there was a significant interaction between age and parity on nitrate/nitrite excretion in virgin and previously pregnant HAF rats, aged 10 and 16 months. Nitrate/nitrite excretion decreased in virgin HAF, aged 16 months, compared with virgin and previously pregnant HAF at 10 months. However, by 16 months of age, previous pregnancy increased nitrate/nitrite excretion in HAF to levels similar to HAF groups at 10 months of age.

Intrarenal Gene Expression Analyses

To determine if there were differences in intrarenal gene expression that could contribute to differences in MAP among virgin and previously pregnant control and HAF rats, 16 months of age, we evaluated mRNA expression of eNOS, and components of the renin-angiotensin system (RAS) and the ET system. As shown in Table 4, eNOS expression was decreased in virgin HAFs compared with controls, whereas pregnancy normalized eNOS in HAF. In components of the RAS, renin, and AT1aR mRNA were lower, and angiotensinogen mRNA was higher in both HAF groups. Pregnancy tended to further reduce AT1aR expression in HAF, and ACE was also lower in previously pregnant HAF than virgin HAF. Intrarenal pre-pro-ET-1 mRNA was higher in virgin HAF than controls or previously pregnant HAF, ET_AR expression was lower in previously pregnant HAF compared with virgin HAF,

Table 3. BWs and Body Composition of Virgin and Previously Pregnant HAF Rats at 10 and 16 Months of Age

Parameter	HAF, 10 mo Virgin	HAF, 10 mo Previously Pregnant	HAF, 16 mo Virgin	HAF, 16 mo Previously Pregnant	Interaction	Parity	Age
BW, g	399.5±6.4	392.3±5.2	427.8±7.4	392.5±16.7	*P*=0.16	*P*=0.02	*P*=0.02
Fat mass, g	56.2±3.8	54.7±4.2	81.6±8.7	80.5±7.8	*P*=0.95	*P*=0.82	*P*<0.01
Lean mass, g	317.2±5.4	315.6±5.6	321.1±10.5	321.1±10.5	*P*=0.34	*P*=0.25	*P*=0.64
Fat mass/BW ratio	0.14±0.01	0.14±0.01	0.19±0.02	0.20±0.01	*P*=0.78	*P*=0.86	*P*<0.01
Lean mass/BW ratio	0.80±0.01	0.80±0.01	0.75±0.02	0.75±0.01	*P*=0.71	*P*=0.75	*P*<0.01

Values represent mean±SEM. Fat and lean masses were determined by Echo-MRI, as described in Methods. Statistical analyses were performed by 2-way ANOVA; significance was defined as $P<0.05$. BW indicates body weight; HAF, hyperandrogenemic female; and MRI, magnetic resonance imaging.

but ET_BR expression was lower in virgin HAF than controls or previously pregnant HAF.

Discussion

In this study, we tested the hypothesis that previous pregnancy would protect HAF rats from age-related hypertension. In fact, we found that (1) previous pregnancy in aging (16 months) HAF rats attenuated the hypertension found in age-matched virgins; (2) along with the reduction in blood pressure, previously pregnant HAF exhibit upregulation of intrarenal eNOS and ET_B receptor mRNA, along with downregulation of renin, ET_AR, and AT1aR mRNA; (3) similarly, previously pregnant HAF rats exhibited a reduction in proteinuria and an increase in nitrate/nitrite excretion compared with virgin HAF rats; (4) before cessation of estrous cycling (10 months of age), there were no differences in blood pressure, proteinuria, or nitrate/nitrite excretion in HAF rats regardless of pregnancy status; (5) male and female offspring born to HAF rats exhibited intrauterine growth restriction with lower birth weights, compared with offspring born to control females.

The HAF rat is a well-characterized model that mimics many of the symptoms of women with PCOS, including insulin resistance, hyperlipidemia, and elevated blood pressure as early as 14 to 16 weeks of age.[19] However, this is the first study, to our knowledge, to address the cardiovascular-metabolic consequences of pregnancy using the HAF model. Common rodent models that seek to study the outcomes of hyperandrogenemic pregnancy on dams or the offspring inject dams with testosterone beginning on gestational days 15 to 17 only, and at doses that are significantly higher than the dihydrotestosterone doses we use for the HAF model (0.5 mg/kg per day testosterone versus 0.25–0.33 µg/kg per day dihydrotestosterone).[27,28] Our HAF rats are treated with dihydrotestosterone beginning at 4 weeks of age (prepubertal) and throughout pregnancy and lactation. Thus, the HAF model better mimics pregnancy in women with PCOS than the late gestation androgen model.

In studying the potential changes in gene expression that could contribute to hypertension in virgin HAF rats and protection seen in previously pregnant HAF, we measured mRNA expression of both vasodilators and vasoconstrictors. We found that in previously pregnant HAF rats, there was an increase in eNOS mRNA expression that was supported by an increase in nitrate/nitrite excretion, an index of NO. Whether eNOS activity is increased in previously pregnant rats HAF will need to be determined in future studies. However, we found previously that the components of the NADPH oxidase system are upregulated in young HAF rats.[19] Since aging is associated with increased oxidative stress, it is also possible then that in aging virgin HAF rats, any NO being produced may be scavenged by superoxide, produced by NADPH oxidase, thus reducing the bioavailability of NO to cause vasodilation and contributing to their elevated blood pressure. Whether oxidative stress increases with aging in virgin HAF rats to a greater extent than in previously pregnant HAF will also need to be determined to further identify the mechanisms responsible for their hypertension.

Young women with PCOS have increased plasma renin levels and activity[29,30] and increased plasma prorenin.[31] In contrast, in the present study in the aging HAF rats, we found that intrarenal renin mRNA expression was decreased in both virgin and previously pregnant rats. Androgens are known to upregulate intrarenal angiotensinogen, and we have shown previously that angiotensinogen was elevated by 10-fold at 14 to 16 weeks of age in virgin HAF rats.[19,32] In the present study, angiotensinogen was also increased by 8- to 9-fold but was similar in both HAF groups. Renin is the rate-limiting step in the synthesis of Ang II (angiotensin II), but if renin is not working at Vmax, then the lower levels of renin may be offset by the significant increases in angiotensinogen substrate that would support higher Ang II levels in the HAF rats. In young PCOS women, ACE gene polymorphism was associated with adverse metabolic comorbidities.[33,34] In the present study, intrarenal ACE mRNA tended to be decreased in previously pregnant HAF rats compared with virgins. Similarly, intrarenal AT1aR mRNA expression was decreased significantly in previously pregnant HAF rats compared with virgins. Enalapril (ACE inhibitor) and telmisartan (AT_1R antagonist) were shown by others to cause significant decreases in BP in HAF rats or PCOS women, respectively.[25,35] Taken together, these data support that the RAS may contribute to the elevated blood pressure in HAF rats, but whether virgin HAF has higher Ang II than previously pregnant rats needs to be determined. Previously pregnant HAF rats may also have higher levels of the vasodilator arm of the RAS, Ang(1–7) or AT2R, that could contribute to their attenuated blood pressure compared with virgins, and will also need to be determined.

Young women with PCOS and female to male transsexuals have elevated levels of endothelin.[36,37] There are no studies to our knowledge on the levels of ET-1 in postmenopausal PCOS women. Alexander et al[38] reported that Ang II stimulates intrarenal ET-1 production. Androgens then may directly

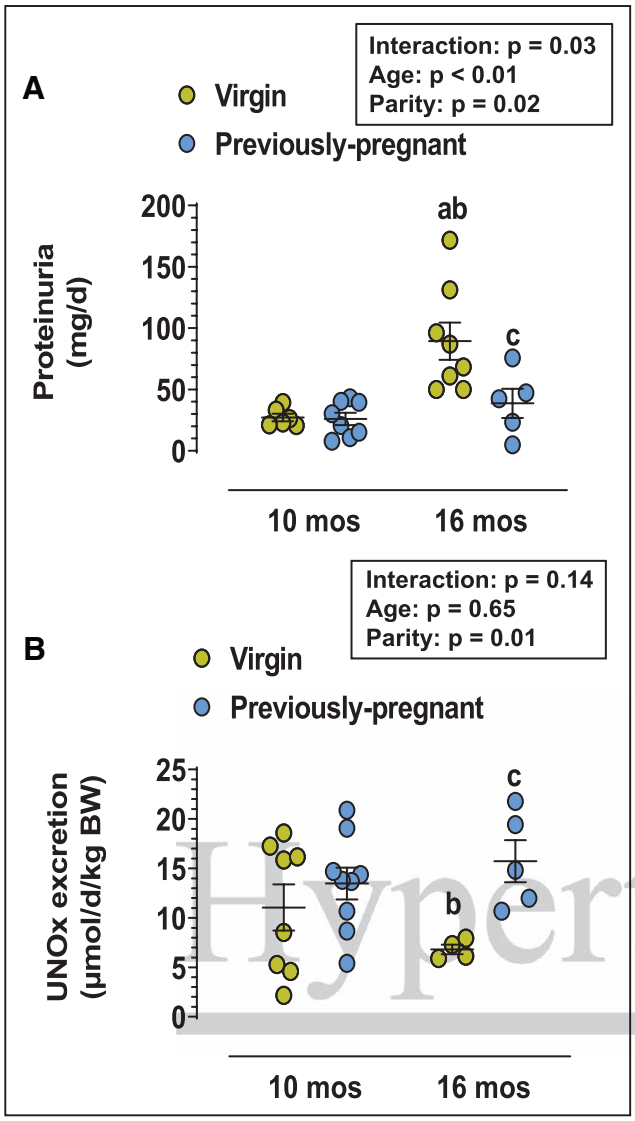

Figure 3. Proteinuria and nitrate/nitrite excretion in virgin and previously pregnant hyperandrogenemic female rats at 10 and 16 mo of age. Proteinuria (**A**) and nitrate/nitrite (UNOx) (**B**) excretion in virgin and previously pregnant hyperandrogenemic female (HAF) rats at 10 and 16 mo of age. **A**, Protein excretion was not different between previously pregnant and virgin HAF rats at 10 mo of age. At 16 mo, protein excretion was higher in virgin HAF compared with all HAF rats at 10 mo. Proteinuria in previously pregnant HAF at 16 mo was similar to HAF rats at 10 mo and was lower than age-matched virgin HAF rats. Statistical significance was determined by 2-way ANOVA with Fisher LSD post hoc test and defined as $P \leq 0.05$. **B**, Nitrate/nitrite excretion was not different in virgin and previously pregnant HAF rats at 10 mo of age, or virgin HAF rats at 16 mo of age. However, nitrate/nitrate excretion was higher in previously pregnant HAF, aged 16 mo, than age-matched virgin HAF rats. Statistical significance was determined by 2-way ANOVA with Fisher LSD post hoc test and defined as $P \leq 0.05$. **A**, $P \leq 0.05$, compared with virgin HAF rats, 10 mo; **B**, $P \leq 0.05$, compared with previously pregnant HAF, 10 mo; **C**, $P \leq 0.05$, compared with virgin HAF, 16 mo. BW indicates body weight.

stimulate endothelin synthesis or may stimulate the RAS to increase endothelin, thus leading to the expression of 2 powerful vasoconstrictors that could impact BP in women with PCOS.[39] In the present study, there were no differences in levels of plasma dihydrotestosterone between the virgin and previously pregnant HAF rats, and pre-pro-ET-1 mRNA expression was upregulated to similar levels in both groups as well, suggesting that intrarenal ET-1 may be similar in the groups. However, intrarenal ET_AR expression was lower and ET_BR expression was higher in previously pregnant HAF, both suggesting these differences could contribute to the higher blood pressure in virgin HAF. Interestingly, Usselman et al[32,37] reported that young PCOS women have elevated ET-1 that endothelial vasodilation via ET_BR predominated but is attenuated compared with controls and that the effect was independent of NO, since nitro-L-arginine methyl esther had no effect on vasodilatory response to ET-1. Additional studies will be necessary to determine the full contribution of endothelin and endothelin receptors to the age-related hypertension in HAF rats.

One interesting finding for the present studies is that the difference in hypertension between previously pregnant and virgin HAF rats does not occur until after cessation of estrous cycling and with advanced aging. At 10 months of age, when the rats are still cycling, the blood pressure, although elevated compared with controls, was not different between previously pregnant and virgin HAF rats. We have shown previously that the HAF rat model ceases to cycle by 13 months of age.[22] If loss of estrogens in the face of increased androgens was the mechanism by which blood pressure increased with aging in the HAF model, one would expect that blood pressure would also rise in previously pregnant HAF rats that have also stopped estrous cycling. Thus, it is not likely that estrogens played a role in the age-related protection against the increase in blood pressure in the previously pregnant HAF rats.

Women with PCOS often have difficulty becoming pregnant and require assisted reproduction.[40] Just as in PCOS women, our HAF rat model also has reproductive difficulties with only 60% of those placed with a male becoming pregnant, compared with >99% of control Sprague-Dawley rats that become pregnant. Another consequence of hyperandrogenemia during pregnancy was the lower birth weights of both male and female offspring of the HAF rats. In women with PCOS, their children are born either small for gestational age,[41,42] as in our study, or large for gestational age.[40] If PCOS women have elevated glucose associated with obesity and insulin resistance, their children may be born large for gestational age.[40] Gopalakrishnan et al[27] reported that testosterone supplements given from day 15 to 19 of pregnancy in rats caused a reduction in both placental weights and fetal weights and was associated with increased expression of markers of hypoxia, such as hypoxia-inducible factor 1α, along with reductions in uterine blood flow.[27] Thus, the presence of elevated androgens in the HAF rats may have contributed to the lower birth weights in their offspring.

We have shown previously that our model of hyperandrogenemia has insulin resistance (elevated insulin levels) but no increase in fasting glucose both as young adults and following estrous cycling,[19,22] so the HAF rats are not diabetic. We have not measured insulin or glucose handling during pregnancy in the HAF model, but since the offspring are born small for gestational age, it is not likely the dams have gestational hyperglycemia. We also have not measured blood pressure during the pregnancy in our HAF rats. However, it is also not likely that pregnancy in these rats is associated with preeclampsia-like symptoms, otherwise the dams should have early CVD and hypertension, as is common for women with preeclampsia.[43] In this regard, the HAF rat model may be similar to

Table 4. Intrarenal mRNA Expression of eNOS, Components of the Renin-Angiotensin System and Endothelin System in Virgin and Previously Pregnant Control Rats, Aged 16 Months, Compared With Age-Matched Virgin and Previously Pregnant HAF Rats

Renal mRNA Expression ($\Delta\Delta C_T$/ Geometric Mean of housekeeping genes)	Control		HAF	
	Virgin (n=6)	Previously Pregnant (n=7)	Virgin (n=12)	Previously Pregnant (n=9)
eNOS (NOS3)	1.00±0.05	0.98±0.08	0.82±0.04*,†	1.03±0.04‡
Renin	1.00±0.13	0.62±0.07*	0.48±0.06*	0.57±0.09*
Angiotensinogen	1.00±0.13	0.87±0.08	8.16±1.30*,†	9.31±1.30*,†
ACE	1.00±0.08	0.97±0.06	1.10±0.08	0.82±0.09‡
AT1aR	1.00±0.05	0.93±0.07	0.69±0.05*,†	0.52±0.03*,†,‡
Pre-pro-ET-1	1.00±0.07	1.05±0.07	1.68±0.20*,†	1.45±0.10
ET_AR	1.00±0.06	1.00±0.08	1.08±0.08	0.91±0.06‡
ET_BR	1.00±0.05	0.96±0.07	0.80±0.02*,†	0.94±0.05‡

Expression values were normalized to the geometric mean of 4 housekeeping genes, as described in Methods in the Data Supplement. Values represent mean±SEM. Statistical analyses were performed using 2-way ANOVA followed by uncorrected Fisher LSD test; significance was defined as $P \leq 0.05$. ACE indicates angiotensin I–converting enzyme; AT1aR, angiotensin 1a receptor; eNOS, endothelial nitric oxide synthase; ET_AR, endothelin A receptor; ET_BR, endothelin B receptor; HAF, hyperandrogenemic female; and pre-pro-ET-1, pre-pro-endothelin-1.

*$P \leq 0.05$, compared with virgin controls.

†$P \leq 0.05$, compared with previously pregnant controls.

‡$P \leq 0.05$, compared with virgin HAF rats.

the spontaneously hypertensive rat that is hypertensive before pregnancy but exhibits a reduction in blood pressure in late pregnancy,[44] and multiple pregnancies have no adverse effect on their renal function or blood pressure.[45] Similarly, Hu et al[10] performed ambulatory blood pressure measurements in PCOS and control women as they progressed throughout their pregnancies. They found that PCOS women had higher blood pressure in the first trimester (11–13 weeks gestation) than control women, but there was no gestational effect, that is, pregnancy per se did not further increase blood pressure.[10] In addition, the average blood pressures in the PCOS women did not reach the levels required for treatment at that time (Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure).[46]

To our knowledge, there are no studies in which the amounts of fat mass and lean mass were measured in PCOS women, prepregnancy, and postpregnancy. We performed these studies in our HAF dams to determine if the pregnancy itself reduced the amount of body fat mass compared with the virgin HAF controls. It was interesting that the levels of fat mass/body weight ratio increased and the lean mass/body ratio decreased so significantly during the time of pregnancy in the HAF and control rats. However, these differences in fat and lean masses observed when the rats were young were not present at 10 and 16 months of age. Thus, the data do not support a role for improved body composition during pregnancy as mediating the lower blood pressure in previously pregnant HAF rats.

One caveat of the present study is that ≈60% of the HAF rats, when placed with a male, became pregnant. Thus, it is possible that the 60% had a more favorable cardiovascular/reproductive system that allowed them to become pregnant and thus with aging also exhibited lower blood pressure. However, there were no differences in the prepregnancy body weights or body composition, lean or fat mass, between the HAF rats, whether they were scheduled to be mated or remain virgins, that could account for the difficulty in becoming pregnant. Female rats can become pseudopregnant, but we have no evidence that this occurred in HAF rats, and only rats that became pregnant and delivered pups were included in the present study.

Perspectives

In the present study, we tested the hypothesis that previous pregnancy would protect against age-related hypertension in our model of PCOS. Despite no differences in dihydrotestosterone levels, body composition, or insulin resistance, hypertension was indeed attenuated in previously pregnant HAF rats compared with virgin HAF. The mechanism(s) responsible for the protection may be differential intrarenal regulation of the NO, endothelin, and renin-angiotensin systems.

While some studies show PCOS women are at increased risk to develop preeclampsia during pregnancy and they may take longer to become pregnant, their pregnancy outcomes are not different than control women.[8,18,47] Thus studies need to be performed in aging PCOS women who have been pregnant and compare them to PCOS women who have never been pregnant to determine their relative risks of developing postmenopausal hypertension and CVD compared with the general population. Now that PCOS diagnosis guidelines have been in place >15 years, these studies should be forthcoming, but in addition to verification of pregnancy, the new studies need to also verify that the women studied actually had PCOS as defined by increased androgens during their reproductive years.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This is the first study to evaluate the consequences of prior pregnancy on age-related hypertension in a rat model of hyperandrogenemia that mimics many of the characteristics of women with polycystic ovary syndrome.
- Previous pregnancy attenuated the hypertension in hyperandrogenemic female rats, aged 16 months, compared with virgin controls.
- Mechanisms responsible are likely differential regulation of intrarenal vasoconstrictors/vasodilators.

What Is Relevant?

- There are no studies that have evaluated the consequences of pregnancy on cardiovascular disease and hypertension in aging, postmenopausal women with polycystic ovary syndrome, who have hyperandrogenemia.

Summary

Previous pregnancy protects hyperandrogenemic female rats from age-related hypertension. The mechanisms for the attenuation of the hypertension are not due to different levels of androgens, body composition (fat mass and lean mass), insulin levels, or glucose metabolism but rather are likely due to increased vasodilator systems (eNOS [endothelial nitric oxide synthase], ET_BR [endothelin B receptor], renin-angiotensin system) and attenuation of the renin-angiotensin system and endothelin vasoconstrictor systems.