ANG II-induced hypertension in the VCD mouse model of menopause is prevented by estrogen replacement during perimenopause

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Submitted 24 April 2015; accepted in final form 20 October 2015

Abstract

Menopausal females are resistant to the development of hypertension, and this protection is lost after the onset of menopause, resulting in a sharp increase in disease onset and severity. However, it is unknown how a fluctuating ovarian hormone environment during the transition from perimenopause to menopause impacts the onset of hypertension, and whether interventions during perimenopause prevent disease onset after menopause. A gradual transition to menopause was induced by repeated daily injections of 4-vinylcyclohexene diepoxide (VCD). ANG II (800 ng·kg⁻¹·min⁻¹) was infused into perimenopausal and menopausal female mice for 14 days. A separate cohort of mice received 17β-estradiol replacement. This study indicates that the changing hormonal environment in the VCD model of menopause impacts the severity of ANG II-induced hypertension. These data highlight the utility of the ovary-intact VCD model of menopause as a clinically relevant model to investigate the physiological mechanisms of hypertension that occur in women during the transition into menopause.

Aquaporin-2; glomerular hypertrophy; collecting duct; estrogen

PREAMENOPAUSAL FEMALES are resistant to the development of hypertension, and this protection is lost after the onset of menopause, resulting in a sharp increase in disease incidence and severity (20). Similarly, resistance to ANG II-induced hypertension has been demonstrated in female rodents. Ovariectomy (the abrupt induction of menopause via the removal of gonads) removes this resistance to ANG II, resulting in a robust ANG II-induced hypertensive response (39). Pharmacological inhibition or genetic ablation of estrogen receptors also increases the hypertensive response to ANG II infusion, suggesting that the loss of 17β-estradiol signaling during menopause is in part responsible for the shift in blood pressure regulation (40).

Repeated daily injections of 4-vinylcyclohexene diepoxide (VCD) can be utilized to gradually induce ovarian failure in mice (termed a mouse model of menopause). After ovarian failure (menopause) residual ovarian tissue remains intact in the VCD model and, similar to that in humans, continues to be hormonally active, secreting androgens (14, 21, 25, 33, 38).

In humans, the transition period preceding menopause is termed “perimenopause” and may last up to 10 yr. Perimenopause is characterized by prolonged periods with low levels of estrogen interspersed with periods of high estrogen (35), and cycle lengths become variable. Similar to the progression of menopause in humans, VCD-treated mice display variations in cycle length, decreased circulating estrogen levels, and increased follicle-stimulating hormone levels as they progress from perimenopause into ovarian failure.

The abrupt loss of ovarian tissue in an ovariectomy model eliminates both the perimenopause period and any potential contributions from residual androgen secretion. Thus it is unknown how the transition from perimenopause to menopause impacts the onset of ANG II-induced hypertension and whether interventions during perimenopause can prevent disease onset after menopause.

In this study, we hypothesized that menopause would promote the development of ANG II-induced hypertension in female mice. In contrast, we hypothesized that in perimenopause the ANG II hypertensive response would remain blunted, as mice continue to cycle and produce estrogen. To test this hypothesis we infused ANG II into mice during either perimenopause or menopause and examined blood pressure responses. We included an estrogen replacement group in the menopause study. We examined renal damage, including glomerular hypertrophy, to determine the role of sex hormones in this disease process (17, 30).

METHODS

Animals

Eight-week-old female C57BL/6J mice were purchased from The Jackson Laboratory. Mice were housed in standard polypropylene cages placed in a temperature- and humidity-controlled facility. The mice were maintained on a 12:12-h light-dark cycle (6 AM to 6 PM) and were fed normal (0.25% NaCl; Harlan Teklad, 7013) mouse chow with water available ad libitum. All methods were approved by the University of Arizona Animal Care and Use Committee.

First published October 21, 2015; doi:10.1152/ajpregu.00170.2015.
Table 1. Description of experimental groups/treatments

<table>
<thead>
<tr>
<th>Description</th>
<th>VCD</th>
<th>ANG II</th>
<th>E2</th>
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</thead>
<tbody>
<tr>
<td><strong>Perimenopause study</strong></td>
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<tr>
<td>Control</td>
<td>Cycling</td>
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<tr>
<td>Peri</td>
<td>Impending ovarian failure</td>
<td>+</td>
<td></td>
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<tr>
<td>ANG II</td>
<td>Cycling</td>
<td>Angiotensin infusion</td>
<td>+</td>
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<tr>
<td>Peri/ANG II</td>
<td>Impending ovarian failure</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Menopause study</strong></td>
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<tr>
<td>Control</td>
<td>Cycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meno</td>
<td>Ovarian failure</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ANG II</td>
<td>Cycling</td>
<td>Angiotensin infusion</td>
<td>+</td>
</tr>
<tr>
<td>Meno + ANG II</td>
<td>Ovarian failure</td>
<td>Angiotensin infusion</td>
<td>+</td>
</tr>
<tr>
<td>Meno + ANG II + E2</td>
<td>Ovarian failure</td>
<td>Angiotensin infusion</td>
<td>Estrogen replacement</td>
</tr>
</tbody>
</table>

Description of treatments given to mice: + indicates a substance that was injected or infused. Otherwise, mice were injected/infused with appropriate vehicle. VCD, 4-vinylcyclohexene diepoxide; ANG II, angiotensin II; Peri, perimenopause; Meno, menopause; E2, 17β-estradiol.

**Experimental Protocol**

To determine whether VCD-induced menopause augments the development of ANG II-induced hypertension, female C57BL/6J mice received daily intraperitoneal injections of VCD (Sigma, V3630) at a concentration of 160 mg/kg or sesame oil vehicle for 20 consecutive days. Vaginal cytology was monitored daily to determine the onset of menopause (ovarian failure), defined as 10 consecutive days of diestrus, as previously described (25). All experimental groups from both the perimenopause and the menopause study are listed with treatments in Table 1, and the study designs are shown in Fig. 1. The VCD model of menopause is well characterized, and similar to previous studies in which VCD was given for 20 consecutive days (21, 22), the average day of ovarian failure in VCD-treated mice occurred on day 54 ± 0.5 days (Meno group); this was not significantly different in the VCD-treated ANG II-infused group (Meno/ANG II), where ovarian failure occurred on day 55 ± 0.5 days.

**Angiotensin II Infusion and Blood Pressure Measurements**

Under isoflurane-induced anesthesia, osmotic minipumps (Alzet, model 1004) containing ANG II (Sigma, A9525; infusion rate 800 ng·kg⁻¹·min⁻¹) were implanted subcutaneously above the right shoulder. In the perimenopause study ANG II was implanted on day 34, 2 wk after the end of VCD dosing and ~20 days before ovarian failure would occur. In the menopause study, ANG II was implanted after all animals had entered menopause.

Blood pressure and heart rate were measured noninvasively via tail cuff (Hatteras Instruments, MC4000) immediately prior to the onset of VCD dosing, immediately prior to ANG II infusion, and after 7 and 14 days of ANG II infusion. All experimental groups are listed in Table 1. All groups were killed after 14 days from the beginning of ANG II infusion.

**Glomerular Area Determination and Mesangial Matrix Expression**

Kidneys were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and sectioned (5 μm). Periodic acid Schiff (PAS) staining was performed on paraffin-embedded kidney sections by the University of Arizona Histology Lab. PAS-stained slides were used to measure glomerular area. To minimize the potential impact of measuring glomeruli on nonidentical planes, 15 cortical images per slide were captured at ×20 magnification, resulting in the analysis of ~45–55 glomeruli per slide, or ~200 glomeruli per treatment group. The mean area (μm²) of each glomerular profile was measured by manually tracing the minimal convex polygon surrounding the glomerular capillary tuft and calculating its area by computerized morphometry using ImageJ software (National Institutes of Health).

To determine mesangial matrix expression, PAS-stained slides were digitally scanned (AperioAT2 Digital Whole Slide Scanner, Leica Biosystems) at ×20 magnification. The minimal convex polygons surrounding the glomerular capillary tuft of all open glomeruli from the resulting images were manually traced and analyzed for percent positive staining with the standard Positive Pixel Count v9 algorithm with the color saturation threshold adjusted to 0.14 (Image Scope v12 viewing software). All analysis was conducted in a blinded fashion.

**Sample Preparation, SDS-PAGE, and Immunoblotting**

In brief, samples were prepared and semiquantitative Western blot analyses were performed on inner medullary, cortex, and whole kidney samples as previously described, and full details are given in previous studies (4, 5). Aquaporin-2 (AQP2) protein was quantified with rabbit anti-AQP2 antibody (1:500 dilution; Novus Biologicals, NB110-74682) overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2,000 dilution; Cell Signaling 7074) for 1 h at room temperature. AQP2 antibodies detect both the 29-kDa AQP2 protein and the 35- to 50-kDa complex of AQP2 glycosylated proteins. Images were obtained and quantified with Quantity One software (Bio-Rad), with intensity values normalized such that the mean value of the control group was defined as 100% expression. All quantification was performed on samples run on the same gel.

**Statistics**

Data were analyzed by one-way ANOVA followed by Tukey’s multiple-comparison post hoc test or Student’s t-test with Graph Pad Prism Software v6 (GraphPad Software, La Jolla, CA) to identify differences between groups. In all tests, P ≤ 0.05 was considered significant. Results are presented as means ± SE.

**RESULTS**

**Menopause Study**

ANG II infusion in VCD-menopause model induces a significant increase in mean arterial pressure, which is prevented by 17β-estradiol replacement. The VCD model of menopause mimics a female’s natural progression into ovarian senescence.
ANG II was infused for 14 days into cycling mice (ANG II), VCD-induced menopausal mice (Meno/ANG II), or VCD-induced menopausal mice with 17β-estradiol replacement (Meno/ANG II/E2) (see Fig. 1 for study design). Infusing ANG II into menopausal mice resulted in a significant increase in mean arterial pressure (MAP) compared with ANG II infusion in cycling females (Fig. 2). 17β-Estradiol replacement prevented the ANG II-induced increase in MAP. In the absence of ANG II, VCD induction of menopause had no impact on blood pressure. Heart rate was not significantly changed in any group.

ANG II-induced glomerular hypertrophy in female mice occurs independently of hormonal status. Glomerular hypertrophy is an early pathophysiological adaptation in response to ANG II-induced hypertension. Therefore, we determined the glomerular areas in PAS-stained renal sections to assess whether the onset of menopause altered the susceptibility of female mice to glomerular hypertrophy induced by ANG II. Quantification with ImageJ software demonstrated that ANG II infusion produced significant glomerular hypertrophy in all groups (Fig. 3). Neither menopause nor 17β-estradiol replacement impacted the degree of ANG II-induced hypertrophy, suggesting that ANG II is acting independently of blood pressure or hormonal status.

Mesangial matrix expansion does not occur in female mice during ANG II-induced hypertension. The presence of mesangial matrix expansion in glomeruli is a well-characterized marker of early hypertensive renal disease, often accompanied by glomerulosclerosis and a decline in renal function. Therefore, glomerular composition was analyzed from digitally scanned PAS-stained renal sections for mesangial matrix positive staining. Regardless of blood pressure or hormonal status, ANG II infusion did not increase the degree of glomerular mesangial matrix expression in any group (Fig. 4).

Aquaporin-2 protein expression decreases in response to ANG II-induced hypertension. Elevated ANG II levels have been demonstrated to alter the expression levels of many renal proteins involved in the transport of salt and water. Previous studies identified a reduction in the expression of collecting
duct protein AQP2 following ANG II infusion (16). Here we examined the expression of AQP2 in ANG II-infused cycling and menopausal females. As shown in Fig. 5A cortical AQP2 protein expression was significantly reduced in ANG II-infused menopausal mice (control 100 ± 5% vs. Meno/ANG II 63 ± 6%, $P \leq 0.05$), while medullary AQP2 expression was unchanged (Fig. 5B). In ANG II-infused menopausal mice treated with 17β-estradiol, AQP2 protein expression was not different from control (Fig. 5C) (control 100 ± 14% vs. Meno/ANG II/E2 100 ± 12%, not significant).

### Perimenopause Study

Perimenopausal female mice demonstrate blunted hypertensive response to ANG II infusion compared with menopausal mice. As previously described, a major strength of the VCD model of menopause is perimenopause, a transition period immediately preceding menopause. In a separate cohort of mice, ANG II was infused into VCD-treated female mice during perimenopause. As shown in Fig. 6, the MAP after ANG II infusion was similar in the cycling and perimenopausal mice, suggesting that resistance to ANG II-induced hypertension is retained during the perimenopause transition.

### DISCUSSION

The results of this study solidify the ovary-intact VCD mouse model of menopause as a clinically relevant model that can be used to identify the physiological progression of hypertension in females. The major findings of the present study are as follows: 1) Perimenopausal mice produce a blunted hyper-

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**Fig. 4.** Effect of ANG II infusion on mesangial matrix expansion in menopausal mice. Glomeruli from digitally scanned PAS-stained renal sections were analyzed for positive staining with Image Scope software. Quantification shows that glomerular mesangial matrix expansion was not significantly different in any group. $n = 3$ mice/group.

**Fig. 5.** Aquaporin-2 (AQP2) protein expression is decreased in kidneys of ANG II-infused menopausal mice. ANG II was infused into menopausal and cycling mice. A–C: protein abundance was analyzed by Western blot. Each lane represents a homogenate from an individual mouse. A and B: representative immunoblots examining AQP2 protein expression in renal cortex (A) and renal inner medulla (B). In a separate cohort of mice, 17β-estradiol replacement was given to Meno/ANG II mice, and whole kidney samples were prepared. C: immunoblot demonstrating that decrease in AQP2 protein expression is prevented by 17β-estradiol replacement in ANG II-infused menopausal mice. D–F: densitometric analysis of Western blots in A–C. Values were normalized to a control (100%) to facilitate comparison. *Significant difference between control and ANG II-infused menopausal (MAII) mice ($P \leq 0.05$).

**Fig. 6.** Effect of ANG II infusion on hemodynamic responses in perimenopausal mice. A: MAP response to ANG II was not different between perimenopausal mice (Peri) and cycling mice (Con). B: changes in MAP are presented as delta change ($\Delta$ change) from day 0 to day 14 after onset of ANG II infusion. Results are expressed as means ± SE; $n = 10$ mice/group. *$P \leq 0.05$ vs. control.
Estrogens cease as follicle depletion occurs; however, residual male mice, which can be prevented by castration (10, 12, 32). Estrogens are known to promote ANG II-induced hypertension in elsewhere (31). Estrogen’s effect is known to be partially blood pressure regulation have been extensively reviewed (8, 20).

The mechanism for female resistance to hypertension before menopause is unknown. In males, the majority of ANG II’s prohypertensive responses are suggested to be mediated through the angiotensin type 1 receptor (AT1). In contrast, females preferentially express the angiotensin type 2 receptor (AT2), which could partially mediate 17β-estradiol’s ability to suppress ANG II-induced hypertension in vivo (1, 2). Aging decreases AT2 receptor expression in favor of AT1 receptors in female mice, and in doing so shifts the pressure natriuresis curve to the right, rendering females more susceptible to the effects of ANG II (11, 27, 28). Further studies are needed in the VCD model of menopause to examine these pathways.

An important component of the VCD-treatment model of menopause is the inclusion of the perimenopause transition period prior to ovarian failure (14, 21, 26, 33). Previous studies have examined menopause as a pre- vs. postmenopausal environment; now we have the ability to investigate how disease progression occurs during more physiological changes to the hormonal milieu. Here we show that during perimenopause ANG II infusion does not induce hypertension. These results build upon our previous studies in which we investigated how the transition from perimenopause to menopause impacted the development of the metabolic syndrome (34). We demonstrated that VCD-treated mice given a high-fat diet gained significantly more weight and had impaired glucose tolerance compared with cycling female mice on a high-fat diet. Diabetic kidney damage also accelerates more rapidly in female mice when diabetes is induced during menopause compared with perimenopause (15). Together our data suggest that inclusion of the perimenopause period for mechanistic evaluations will likely be a useful tool to investigate the subsequent susceptibility to disease onset and progression.

A classic early pathological response to hypertension is glomerular hypertrophy (9). Significant hypertrophy occurs in several models of hypertension including the ANG II, 5/6 nephrectomy, and salt-sensitive hypertension models (3, 23, 17, 30). Indeed, the concentration of ANG II in Bowman’s space has been shown to be 1,000-fold higher than in the systemic circulation (36). Additionally, a recent study in male Dahl salt-sensitive rats suggests a link between glomerular hypertrophy and oxidative stress (3). However, no sex differences have been reported for the role of ANG II in glomerular hypertrophy, and our previous data suggested that ANG II-induced hypertrophy is independent of sex (29). In this study, we show that ANG II-induced glomerular hypertrophy was independent of blood pressure or hormonal status, suggesting a local role for ANG II in modifying renal architecture, separate from changes in blood pressure. Indeed, angiotensin receptor blocker administration in male Ren-2 transgenic rats can partially prevent glomerular volume expansion in the 5/6 nephrectomy model of hypertension (17), while the combination therapy of a calcium channel blocker (azelnidipine) and an angiotensin receptor blocker (olmesartan) given to male salt-sensitive rats reduces mean glomerular area to a greater degree than when either is given alone (30). Interestingly, blood pressures in the treatment groups were also reduced, making it difficult to determine whether the reduction in glomerular area...
was a result of blood pressure normalization or a function of angiotensin receptor antagonism. Our results would indicate that the reduction in blood pressure per se is not entirely responsible for the glomerular protection. However, since the present study was conducted in female mice, the contribution of sex or species differences in this process cannot be excluded and remains to be fully elucidated.

Blood volume regulation and the role of renal sodium and water excretion is another important component of long-term blood pressure control. AQ2P protein expression in the principal cells of the collecting duct is tightly coupled to water balance (37), and thus it has been speculated that its expression and trafficking may play a role in hypertension. Klein et al. reported that AQ2P protein expression was decreased after the onset of ANG II-induced hypertension; they also observed a decrease in AQ2P expression in a norepinephrine-induced hypertension model (16). The increase in blood pressure was similar in all treatment groups, with no change in vasopressin levels, suggesting that the decline in protein expression was a response to the increased blood pressure. In contrast, others have suggested that AQ2P expression increases in hypertension models and could contribute to the disease progression. In the DOCA-salt and spontaneously hypertensive rat (SHR) models of hypertension, AQ2P levels were increased relative to nonhypertensive controls (6, 19). Additionally, antagonism of the AT1 receptor during dDAVP treatment significantly reduces AQ2P expression in NaCl-restricted rats (18). Here we suggest that AQ2P protein expression was inversely associated with blood pressure. In ANG II-infused menopausal mice demonstrating a robust hypertensive response, AQ2P expression was significantly decreased. However, AQ2P expression levels were restored to control levels when blood pressure was normalized in the ANG II-infused menopausal mice after 17β-estradiol replacement. Little is known regarding the effects of estrogen on AQ2P protein expression or recycling during hypertension. However, Armando et al. demonstrated that female mice express fewer renal AT1 receptors compared with males and that ovariectomy alters the AT1;AT2 receptor balance in a segment-specific manner, which may manifest in fundamental differences in ANG II/vasopressin-induced AQ2P regulation (1). However, considerable ambiguity regarding the cause/effect relationship between sex hormones, arginine-vasopressin release, and AQ2P protein expression during ANG II-induced hypertension exists, and additional investigations are warranted.

Perspectives and Significance

The present study demonstrates the utility of the VCD-treatment model of menopause for the study of hypertension and associated renal disease. Female mice developed an augmented ANG II-induced hypertension as they progressed into menopause, similar to the increase in hypertension seen in the human population. The timing of when to administer preventative therapies in women is crucial to disease progression. The inclusion of the perimenopause transition period in the VCD model of menopause makes a useful intervention point for future therapeutic studies, with the goal of understanding mechanistically how to prevent the progression and severity of hypertension and subsequent renal disease in postmenopausal women.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Chip Brosius for helpful advice in analyzing mesangial matrix expansion and M. Snyder and Dr. Surabhi Chandra for technical assistance.

GRANTS

This work was funded by National Institutes of Health Grants DK-073611 (H.L. Brooks) and T32 HL-007249 (D. P. Pollow, Jr.), an American Heart Association Predoctoral Fellowship (D. P. Pollow, Jr.), University of Arizona Sarver Heart Center Heart Disease in Women Research Grant (H. L. Brooks/J. Konhilas and D. P. Pollow, Jr.), and the Stevie and Karl Eller Achievement Rewards for College Scientists award (D. P. Pollow, Jr.).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


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