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Sildenafil Citrate Does Not Reprogram Risk of Hypertension and Chronic Kidney Disease in Offspring of Preeclamptic Pregnancies in the Dahl SS/Jr Rat

Hannah R. Turbeville, Ashley C. Johnson, Michael R. Garrett, and Jennifer M. Sasser

Abstract

Background Preeclampsia is a disorder of pregnancy with accompanying high disease and economic burdens in the United States. Evidence supporting longstanding effects of preeclampsia on the offspring of affected pregnancies is high, but the effects of current antihypertensive therapies for preeclampsia on cardio-renal outcomes are largely unknown. The purpose of this study was to test the hypothesis that sildenafil citrate, a phosphodiesterase-5 inhibitor, reprograms the risk of hypertension and kidney disease in offspring of preeclamptic pregnancies by altering responses to secondary stressors.

Methods Dahl SS/Jr rats on a 0.3% NaCl diet were mated. At gestational day 10, pregnant dams were randomized to vehicle diet or diet with sildenafil (50 mg/kg per day), which was continued until birth. Pups were weaned at 4 weeks of age and allowed to age on a 0.3% NaCl diet until 3 months of age. At this point, pups were randomized into three groups: baseline or no intervention, 2% NaCl diet challenge for 4 weeks, or a subpressor infusion of angiotensin II (200 ng/kg per minute) for 2 weeks.

Results There were no differences among maternal treatment groups at baseline. Upon introduction of 2% NaCl diet, male offspring of sildenafil-treated dams exhibited an attenuated rise in BP; however, this protection was not observed during angiotensin II infusion.

Conclusions Our findings indicate that intrapartum sildenafil does not reprogram the risk of hypertension and kidney disease in offspring of preeclamptic pregnancies.

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Introduction

Preeclampsia is a disorder of pregnancy affecting up to 8% of pregnancies worldwide (1). It emerges after 20 weeks of pregnancy and typically results in new-onset hypertension and proteinuria, although thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, and vision problems are also included as diagnostic symptoms (1). Although symptoms were originally thought to subside with delivery, it is increasingly apparent that preeclampsia conveys significant risk to both mother and child beyond the postpartum period (2–6). For pregnancies complicated by preeclampsia, delivery occurs earlier and with more adverse events than those of uncomplicated pregnancies or with preexisting hypertension. These adverse events lead to a high economic burden, with mean maternal and infant health care costs for preeclampsia at $41,790 versus $13,187 for uncomplicated pregnancies and $24,182 for women with preexisting hypertension (7).

Pregnancy represents a critical window of development and physiologic programming for the fetus. Barker and colleagues showed that children born of pregnancies complicated by preeclampsia or low birth weight exhibit higher BP during childhood and into adulthood (8,9). This hypothesis, named the Developmental Origins of Health and Disease theory, proposes that an adverse intrauterine environment (insufficient nutrition or blood flow to the fetus) results in epigenetic changes that alter metabolism and physiologic response mechanisms throughout the life of the offspring (10,11). Further studies and meta-analyses showed that children born after preeclamptic pregnancies are at greater risk for vascular diseases such as hypertension and stroke (3,12,13). Although several proposed mechanisms for this link have gained evidentiary support (14–17), the exact nature of this pathogenesis remains unknown, and so no potential therapeutic interventions have been shown.

Despite increasing research for potential preeclampsia treatments, the current standard of care is limited to careful monitoring, limited antihypertensive treatment, magnesium sulfate infusion for prevention of eclamptic seizures, and early delivery (18). Although continued gestation is usually beneficial for fetal development, continued exposure to an intrauterine environment with suboptimal nutrition conveys additional risk (19,20). Experimental therapies currently

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being tested include antioxidants (*e.g.*, aspirin, vitamins C and E), vasodilators (*e.g.*, sildenafil), and inhibition or reduction of soluble fms-like tyrosine kinase 1 (sFlt-1; *e.g.*, infusion of VEGF, apheresis, *etc.*), among others (21–28). Although clinical and/or preclinical studies have demonstrated the beneficial effects of these agents in the short term, long-term safety data is limited on most therapies (18). True randomized, clinical trials are difficult to establish because any untreated control group would not receive the standard of care, and safety concerns for the mother and fetus supersede limitations of trials during pregnancy.

Sildenafil citrate is a phosphodiesterase-5 (PDE-5) inhibitor that prevents degradation of cyclic guanosine monophosphate (cGMP), prolonging the nitric oxide (NO)-cGMP signaling cascade and promoting vasorelaxation (29,30). PDE-5 is expressed in the uterine vasculature, and sildenafil has been shown to improve vasorelaxation of myometrial vessels (31,32). Therapeutic oral doses of sildenafil demonstrate an ability to cause local vasorelaxation to improve fetoplacental perfusion as well as producing only a modest and transient decrease in systemic BP (33). Furthermore, a reduction in endogenous NO production has been identified in preeclampsia, and sildenafil has been shown to attenuate pathogenesis of this disease (34–36). We have previously shown that sildenafil attenuates the maternal phenotype of preeclampsia and results in improved pup growth and increased litter size in the Dahl salt-sensitive S (SS/Jr) rat, an established model of spontaneous, superimposed preeclampsia (24,37). We have also shown that offspring of sildenafil-treated dams exhibit a slight reduction in mean arterial pressure (MAP) in early life, although these prior studies ended at 20 weeks of age (38). The purpose of this study was to extend these findings with longer follow-up and test the hypothesis that sildenafil citrate reprograms the risk of hypertension and kidney disease in offspring of preeclamptic pregnancies by altering responses to secondary stressors.

Materials and Methods

Animals

Dahl SS/Jr rats were obtained from the colony maintained by Dr. Michael Garrett at the University of Mississippi Medical Center. All rats were fed low-salt chow (TD7034, 0.3% NaCl; Harlan Teklad, Madison, WI) and water *ad libitum* on a 12-hour light/dark cycle. Male and female rats were mated in nonconsanguineous groups. Presence of sperm in a vaginal swab was indicative of gestational day 1. Lactating dams and pups were on normal chow until gestational day 10, when dams were stratified into control or sildenafil groups. Littermates were split between groups, and an equivalent level of preexisting renal injury was ensured by measurement of urine protein before males were introduced. Sildenafil (50 mg/kg per day) was mixed with normal chow until delivery, as in prior studies (38). Dams who remained on the normal chow diet without drug introduction are referred to as the vehicle group (VEH). Dams were allowed to give birth spontaneously, and pups were weaned at 4 weeks of age. One male and one female pup per litter were utilized for each study. There is no difference in any intervention provided between groups of offspring. Two distinct subsets of offspring were followed without intervention to either 3 or 6 months of age as a time-course study. The euthanasia of these animals at the prescribed time points for tissue collection precluded the study of a single group of animals over this time. Two subsets distinct from those used in the time-course study were used to test BP response to secondary stressors, including elevated salt diet (2% NaCl, $n=15$) and angiotensin II (AngII; $n=33$) infusion, as described below. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were monitored by the University of Mississippi Medical Center Institutional Animal Care and Use Committee.

BP Measurements

Time-Course Studies. Systolic BP measurements were obtained using the volumetric pressure recording tail-cuff method (CODA 8-channel system; Kent Scientific Corp., Torrington, CT). Animals were trained in restraints alone followed by training in restraints on a heated platform before obtaining sets of no fewer than five valid measurements each on two consecutive days. All valid measurements for each animal were averaged to obtain the final data points.

Secondary Stressor Studies. MAP measurements were obtained using a telemetry method as previously described (39). Rats were implanted with telemetry devices (Data Sciences, Inc., St. Paul, MN) *via* the femoral artery for continual BP monitoring at 10 weeks of age, approximately 10 days before each experiment, allowing 1 week for recovery and 2–3 days for collection of baseline BP data.

Elevated Salt (2% NaCl) Diet

After recovery from telemetry surgery and collection of baseline MAP data, a subset of rats ($n=15$; 1–4 per group) was fed an elevated salt diet (2% NaCl, TD94217; Harlan Teklad) for 4 weeks starting at approximately 12 weeks of age. Twenty-four hour urine was collected *via* metabolic cage before initiation of the 2% salt diet and before euthanasia and tissue collection at the end of the 4-week study.

AngII Infusion

After recovery from telemetry surgery and collection of baseline MAP data, minipumps (model 2002; Alzet) were inserted subcutaneously in a subset of rats ($n=33$; 5–6 per group) starting at approximately 12 weeks of age. AngII (Sigma Aldrich, St. Louis, MO) was diluted in sterile saline and delivered at a subpressor dose of 200 ng/kg per minute for 2 weeks before euthanasia and tissue collection. Twenty-four hour urine was collected *via* metabolic cage before minipump insertion and again before euthanasia.

Urinary Measurements

Rats were placed in metabolic cages with free access to food and water for 24-hour urine collection. Urinary protein excretion was determined by Bradford Assay (Bio-Rad Laboratories). Urinary excretion rates of KIM-1 (1:8 dilution;

Table 1. Normalized counts of all genes studied *via* targeted RNA sequencing in 3-month-old male rats

Gene	Vehicle Male	Sildenafil Male	*P* Value
Agtr1a	0.015	0.012	0.46
Cat	1.041	1.479	0.06
Col3a1	0.104	0.08	0.48
Edn1	0.007	0.009	0.31
Ednra	0.001	0.001	0.73
Ednrb	0.059	0.053	0.51
Gpx2	0.009	0.020	0.07
Gss	0.595	0.864	0.28
Hmox1	0.007	0.006	0.58
Hmox2	Und	Und	—
Hif3a	0.000	0.001	0.02[a]
Il10	Und	Und	—
Il17a	Und	Und	—
Il6	Und	Und	—
Havcr1	0.023	0.033	0.32
Nox4	0.151	0.153	0.96
Nphs1	0.027	0.027	0.97
Lcn2	0.009	0.01	0.77
Nos2	Und	Und	—
Nos3	0.005	0.004	0.28
Pde5a	0.005	0.008	0.17
Nphs2	0.103	0.119	0.53
Prkg2	Und	Und	—
Atp6ap2	0.113	0.157	<0.001[a]
S100a4	0.031	0.026	0.61
Sod1	2.609	2.875	0.53
Sod2	0.627	0.666	0.33
Sod3	0.353	0.508	0.05[a]
Tgfb1	0.019	0.019	>0.99
Timp1	0.033	0.033	>0.99
Tnf	Und	Und	—
Vim	0.141	0.149	0.82

Und, undetectable; —, not applicable.
[a]$P<0.05$ versus vehicle.

Table 2. Normalized counts of all genes studied *via* targeted RNA sequencing in 3-month-old female rats

Gene	Vehicle Female	Sildenafil Female	*P* Value
Agtr1a	0.020	0.023	0.52
Cat	1.039	0.824	0.35
Col3a1	0.087	0.086	0.98
Edn1	0.005	0.007	0.30
Ednra	0.002	0.002	0.66
Ednrb	0.079	0.078	0.86
Gpx2	0.018	0.012	0.15
Gss	0.821	0.713	0.63
Hmox1	0.006	0.004	0.05
Hmox2	Und	Und	—
Hif3a	0.001	0.001	0.11
Il10	Und	Und	—
Il17a	Und	Und	—
Il6	Und	Und	—
Havcr1	0.048	0.025	0.11
Nox4	0.097	0.105	0.85
Nphs1	0.035	0.036	0.96
Lcn2	0.020	0.015	0.49
Nos2	Und	Und	—
Nos3	0.007	0.007	0.81
Pde5a	0.022	0.015	0.45
Nphs2	0.097	0.102	0.89
Prkg2	Und	Und	—
Atp6ap2	0.162	0.131	0.22
S100a4	0.028	0.032	0.69
Sod1	2.156	1.959	0.60
Sod2	0.542	0.582	0.56
Sod3	1.369	1.415	0.92
Tgfb1	0.023	0.026	0.59
Timp1	0.042	0.036	0.53
Tnf	Und	Und	—
Vim	0.173	0.142	0.43

Und, undetectable; —, not applicable.

R&D Systems, Minneapolis, MN) and nephrin (no dilution; ABclonal, Woburn, MA) were quantified *via* commercially available ELISAs. Urine sample dilutions were adjusted as necessary to achieve linear fit for each assay.

Tissue Collection

Rats were anesthetized using isoflurane (5% induction, 2%–3% maintenance; Piramal Healthcare). A terminal blood sample was obtained from the abdominal aorta into heparinized syringes, and organs were subsequently perfused blood-free with saline. The kidneys were removed, dissected into cortical and medullary regions, and snap-frozen in liquid nitrogen for later analysis.

Creatinine Clearance Measurements

Terminal blood samples were centrifuged, and plasma was isolated. Creatinine concentrations were measured in both urine and plasma samples (Vet Axcel Chemistry Analyzer; Alfa Wasserman, West Caldwell, NJ) and then used to calculate creatinine clearance for each animal.

Targeted RNA-Sequencing

Expression analysis was performed on genes involved in inflammation, glomerular function, renal injury, and reactive oxygen species. RNA was isolated from kidney using an automated KingFisher Flex nucleic acid system along with KingFisher Pure RNA Kit. RNA was evaluated for quantity (Nanodrop One and Qubit Fluorimeter) and quality using Qiagen QIAxcel advanced system. The Illumina DesignStudio application (http://designstudio.illumina.com/) was utilized to design custom amplicons across exon-intron boundaries of target genes ($n=32$ gene with one to two probes per gene). The gene target/probes that were designed/used are listed in Supplemental Table 1. On the basis of the DesignStudio output, the TruSeq Targeted RNA Custom Panel Kit was ordered and subsequently utilized to prepare a library for collected RNA samples. The Illumina MiSeq platform allows for analysis of pooled libraries (*e.g.*, $n=96$–384 RNA samples) to be processed at a single time as individual samples will have a unique "barcode." Libraries were sequenced on Illumina MiSeq using MiSeq Reagent Kit v2 (150 cycles). Sequencing reads were demultiplexed and aligned to rn6 genome assembly using RNA Amplicon Application (along with custom panel manifest), available on Illumina BaseSpace Computing Platform. For each gene, counts per million, were normalized to average of counts per million for housekeeping genes to provide normalized measure of expression.

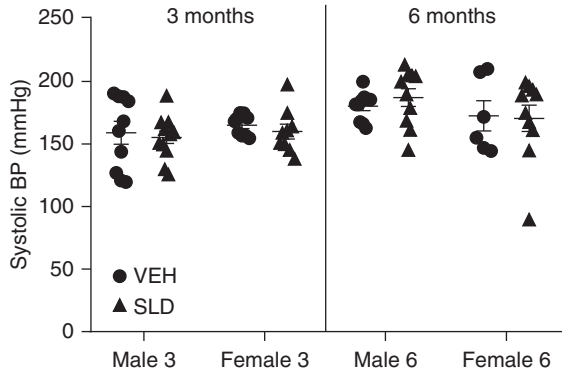

Figure 1. | Maternal sildenafil treatment has no effect on baseline systolic BP at either 3 or 6 months of age. Tail-cuff measurement, n=10–21 per group. SLD, sildenafil; VEH, vehicle.

Statistical Analyses

All data are presented as mean±SEM. Statistical analyses were performed by two-way ANOVA (with repeated measures for telemetry data) followed by Tukey *post hoc* analysis using GraphPad Prism 8.0 (GraphPad, San Diego, CA). t test was utilized for Tables 1–4. Means were considered significantly different if $P<0.05$.

Table 3. Normalized counts of all genes studied *via* targeted RNA sequencing in 6-month-old male rats

Gene	Vehicle Male	Sildenafil Male	*P* Value
Agtr1a	0.016	0.022	0.52
Cat	1.723	1.560	0.76
Col3a1	0.121	0.103	0.50
Edn1	0.012	0.003	0.20
Ednra	0.001	0.002	0.83
Ednrb	0.047	0.064	0.20
Gpx2	0.025	0.020	0.66
Gss	0.605	0.438	0.32
Hmox1	0.005	0.009	0.30
Hmox2	Und	Und	—
Hif3a	0.003	0.000	0.33
Il10	Und	Und	—
Il17a	Und	Und	—
Il6	Und	Und	—
Havcr1	0.057	0.033	0.44
Nox4	0.209	0.332	0.30
Nphs1	0.011	0.026	0.06
Lcn2	0.017	0.011	0.50
Nos2	Und	Und	—
Nos3	0.003	0.005	0.46
Pde5a	0.013	0.005	0.09
Nphs2	0.073	0.101	0.11
Prkg2	Und	Und	—
Atp6ap2	0.167	0.166	0.96
S100a4	0.040	0.073	0.10
Sod1	3.031	3.574	0.54
Sod2	0.778	0.913	0.40
Sod3	0.407	0.330	0.30
Tgfb1	0.021	0.017	0.59
Timp1	0.036	0.031	0.61
Tnf	Und	Und	—
Vim	0.144	0.135	0.80

Und, undetectable; —, not applicable.

Results

Maternal Sildenafil Therapy Does Not Affect Baseline Systolic BP

The primary endpoint of interest in this study was long-term BP in offspring of treated and untreated preeclamptic pregnancies. Although offspring of both VEH-fed and sildenafil-treated dams experienced a significant increase in systolic BP from 3 to 6 months of age as expected, no significant difference was observed among treatment groups at either time point (P for treatment=0.85; Figure 1). The increases seen over time are consistent with previous observations in this model and are unaffected by maternal BP therapy during pregnancy (40).

Age-Related Proteinuria Is Accelerated in Male Offspring of Both Treatment Groups, but Maternal Sildenafil Treatment Attenuates Age-Related Decline in Renal Function

Renal injury and decreases in functional capacity are both consequences of elevated BP as well as drivers of continued increases in BP. In this cohort, glomerular injury is accelerated with age in male offspring as compared to females, as shown by proteinuria. From 3 to 6 months of age, proteinuria increases significantly in males of both treatment groups (P for VEH group <0.001; P for sildenafil group <0.001; Figure 2A), but does not change significantly in females (P for VEH group >0.99; P for sildenafil group =0.93). However, similar differences in nephrin excretion are not present, likely because of the variability seen in all groups at 6 months of age (P for VEH males =0.12; P for sildenafil males =0.21; P for VEH females =0.03; P for sildenafil females =0.53; Figure 2B). Nphs1 is the gene for the protein nephrin, a component of the glomerular filtration barrier, and high expression of this gene indicates maintenance of this part of the glomerulus. Similar to the results shown for nephrinuria, no differences are shown in Nphs1 expression between sexes or treatment groups at either 3 or 6 months of age (Figure 2C). No other markers of renal injury reached significance. Creatinine clearance, a measure of renal function, shows that female offspring and male offspring of sildenafil-treated dams experience no significant decline from 3 to 6 months of age (P for VEH females =0.62; P for sildenafil females >0.99; P for sildenafil males =0.33; Figure 3), whereas male offspring of VEH-fed dams exhibit a significant decline in renal function over this time (P for VEH males <0.001).

Male Offspring of Sildenafil-Treated Dams Exhibit an Attenuated BP Response on a 2% Salt Diet

We hypothesized that maternal BP treatment during pregnancy may improve the response to secondary BP stressors in offspring. MAP rose as expected in offspring of both sexes across all groups on a 2% NaCl diet. However, male offspring of sildenafil-treated dams exhibited a significantly attenuated rise as compare to those of VEH-fed dams (P=0.02), similar to that experienced by female offspring of all treatment groups (Figure 4).

Sex Differences in Renal Injury Persist on a 2% Salt Diet

The sex differences in renal injury measures shown in previous experiments were maintained during 2% salt

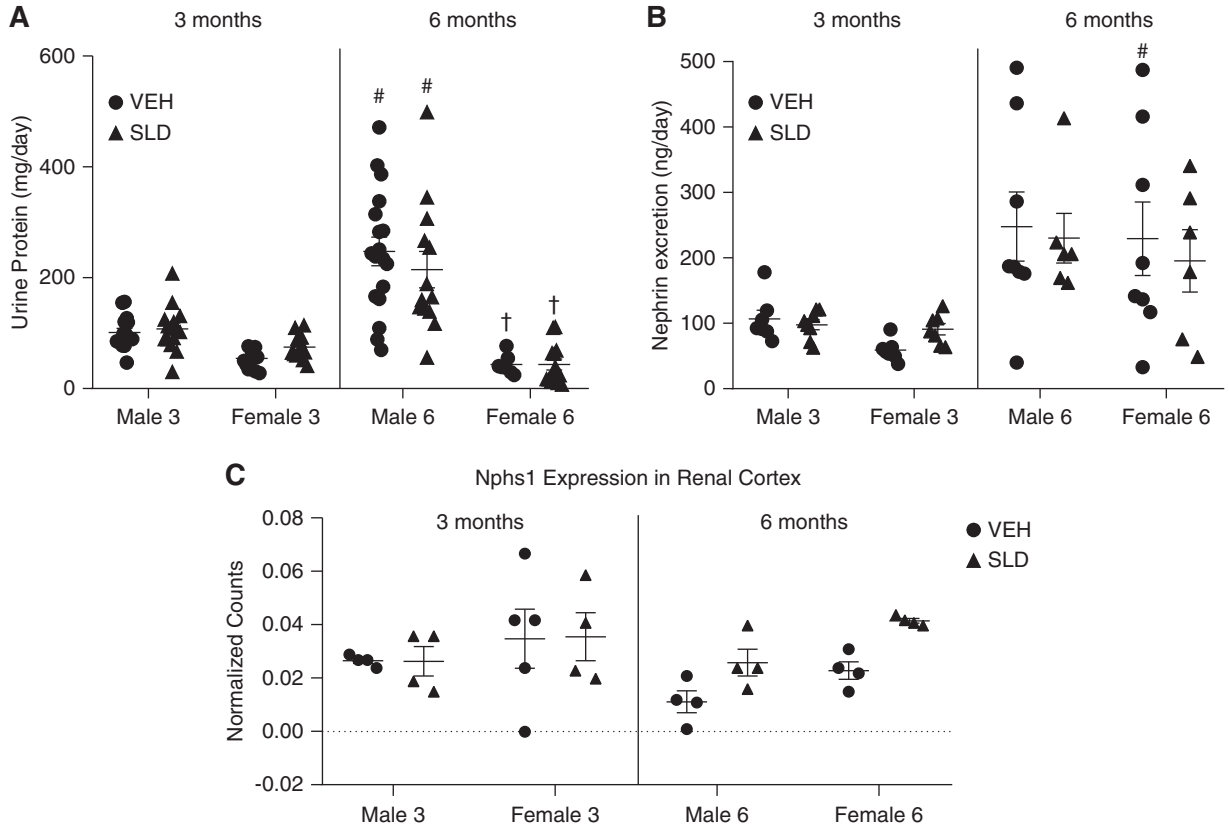

Figure 2. | **Proteinuria is reduced in female offspring of all maternal treatment groups.** (A) Maternal sildenafil treatment does not affect baseline 24-hour proteinuria (n=7–18 per group; $^{\dagger}P$<0.05 versus same age male; $^{\#}P$<0.05 versus same sex at 3 months). (B) Urinary excretion of nephrin is not different between treatment groups or sexes at either 3 or 6 months of age (n=6–8 per group; $^{\#}P$<0.05 versus same sex at 3 months). (C) RNA expression of Nphs1 (nephrin) in the renal cortex is not different between treatment groups or sexes at either 3 or 6 months of age (n=4–5 per group).

feeding. Female offspring of all dams exhibit significantly less proteinuria (P for sex <0.001) and KIM-1 (P for sex <0.001) as compared with male offspring of all dams (Figure 5, A and B). Combined with the BP data, these data indicate that female Dahl SS/Jr rats may have some protection from renal injury as a result of secondary BP stressors, such as elevated salt. However, male offspring of sildenafil-treated dams do not exhibit reduced renal injury or improved renal function despite attenuated BP responses, and no sex differences were observed in creatinine clearance (P for sex =0.16; Figure 5C).

Maternal Sildenafil Therapy Does Not Affect BP Response to AngII Infusion

Next, a second BP stressor was tested in a separate cohort of animals *via* AngII infusion to see if attenuation of BP response extends beyond salt sensitivity. Although MAP increased in all groups as expected, no significant differences were observed between treatment groups of either sex (P for males =0.46; P for females =0.27; Figure 6), indicating a lack of the protective effect of sildenafil suggested by data obtained during the 2% salt diet.

No Differences were Observed in Renal Function or Injury after Chronic AngII Infusion

Similar to the findings of the 2% salt diet study, no differences were observed in creatinine clearance between males and females (P for sex =0.91) or between treatment groups (P for treatment =0.40; Figure 7C). However, sex differences were maintained in urinary excretion of total protein (P for sex <0.001; Figure 7A) and KIM-1 (P for sex =0.003; Figure 7B), similar to previous data shown. These data combined with the aforementioned BP data, suggest that the mechanism of BP response to AngII differs from that to 2% salt diet and is unaffected by maternal treatment.

Discussion

The salient findings from this study show that maternal sildenafil treatment does not affect BP and renal injury or function negatively at baseline. We have shown no differences between maternal treatment groups in systolic BP, proteinuria, nephrinuria (a more sensitive marker of kidney injury [41]), or creatinine clearance under baseline conditions up to 6 months of age. However, significant BP differences emerged upon the introduction of a 2% NaCl diet, where male offspring of sildenafil-treated dams exhibit an attenuated BP response. Such differences were not observed

Table 4. Normalized counts of all genes studied *via* targeted RNA sequencing in 6-month-old female rats

Gene	Vehicle Female	Sildenafil Female	*P* Value
Agtr1a	0.023	0.018	0.22
Cat	0.774	0.706	0.47
Col3a1	0.051	0.053	0.93
Edn1	0.003	0.004	0.84
Ednra	0.001	0.001	0.36
Ednrb	0.064	0.076	0.19
Gpx2	0.012	0.011	0.60
Gss	0.605	0.506	0.27
Hmox1	0.005	0.004	0.43
Hmox2	Und	Und	—
Hif3a	0.001	0.001	0.62
Il10	Und	Und	—
Il17a	Und	Und	—
Il6	Und	Und	—
Havcr1	0.024	0.025	0.89
Nox4	0.132	0.099	0.31
Nphs1	0.023	0.042	0.002[a]
Lcn2	0.016	0.012	0.44
Nos2	Und	Und	—
Nos3	0.005	0.007	0.01[a]
Pde5a	0.012	0.011	0.69
Nphs2	0.079	0.118	0.21
Prkg2	Und	Und	—
Atp6ap2	0.178	0.169	0.76
S100a4	0.046	0.043	0.91
Sod1	2.348	1.682	0.03[a]
Sod2	0.691	0.591	0.22
Sod3	1.290	1.026	0.06
Tgfb1	0.018	0.020	0.54
Timp1	0.031	0.031	0.97
Tnf	Und	Und	—
Vim	0.124	0.139	0.48

Und, undetectable; —, not applicable.
[a]$P<0.05$ versus vehicle.

in the presence of a second BP stressor through infusion of AngII. This suggests that maternal treatment with sildenafil affects BP response to sodium in a sex-specific manner, although specific mechanisms have not been elucidated.

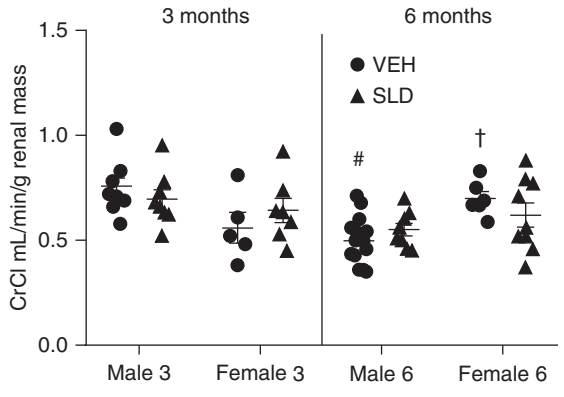

Figure 3. | Renal function does not change significantly with age in female offspring or male offspring of SLD-treated dams. Creatinine clearance (CrCl) is significantly lower in male offspring of VEH dams at 6 months of age versus 3 months of age, whereas no other group experiences a significant decline (n=5–17 per group; $^{\dagger}P<0.05$ versus male; $^{\#}P<0.05$ versus same group at 3 months).

Meta-analysis of studies of individuals exposed to preeclampsia *in utero* yields significant increases in systolic and diastolic BP, as well as body mass index as compared to age-matched controls, regardless of sex or birth weight, from childhood into adolescence (3). Similar results were also found in a birth cohort followed for 20 years postnatal, where *in utero* exposure to preeclampsia conferred a threefold risk of becoming hypertensive by 20 years of age. Although the exact cause of preeclampsia is likely multifactorial, the importance of systemic endothelial dysfunction is well established (42), as well as a reduction in NO production compared with the normal pregnancy state (36,43,44). Endothelial dysfunction is also evident in the offspring of preeclamptic pregnancies (4,45,46). Postnatal loss of vascular density in offspring of preeclamptic pregnancies correlates to maternal levels of antiangiogenic factors such as sFlt-1 and soluble endoglin (47).

Sildenafil was first considered as a treatment for preeclampsia for several reasons: (*1*) selectivity for smaller vascular beds (specifically the uterine vasculature), (*2*) minimal decrease in systolic BP, (*3*) the established role of NO in vasodilation of normal pregnancy, and (*4*) the endothelial dysfunction and reduction in NO bioavailability present in preeclampsia (43,48). PDE-5 inhibitors showed increased endothelium-dependent relaxation in isolated small myometrial arteries (32), with no effect on endothelial-dependent relaxation of omental or placental arteries in preeclampsia (49). Randomized, controlled trials showed beneficial clinical effects, including mild prolongation of pregnancy, reduced uteroplacental arterial resistance, and reduction of maternal MAP (50). Importantly, sildenafil has been shown to significantly reduce the level of sFlt-1 in maternal circulation (34).

Preclinical studies have also shown that treatment with sildenafil is beneficial to offspring of preeclamptic pregnancies. Our laboratory previously showed that sildenafil is effective in attenuating the maternal phenotype in spontaneous, superimposed preeclampsia, including reduction of MAP, reduction of proteinuria, and attenuation of the resulting fetal growth restriction (24,38). More recently, studies on the cognitive impairment exhibited in rodent offspring of preeclamptic models showed that intrapartum sildenafil is also able to attenuate this phenotype (51). Because of sildenafil's proposed benefits to improve fetoplacental blood flow during preeclampsia, we hypothesized that maternal treatment with sildenafil would reprogram the growing fetus to exhibit attenuated hypertension and renal disease during adulthood. Our data do not support the hypothesis that sildenafil improves offspring BP or renal function; however, maternal antihypertensive treatment with sildenafil did attenuate age-related decline in creatinine clearance and reduce salt-sensitive hypertension in male offspring.

We posited that therapies targeting the NO-cGMP signaling pathway, such as sildenafil, would improve regulation of the renin-angiotensin-aldosterone system as well as attenuate salt-sensitive hypertension. However, this is not supported by this study. It is possible that the attenuation of programming effects by intrapartum sildenafil affect a downstream target in the NO pathway, and that although cGMP has the ability to maintain the natriuretic effects of

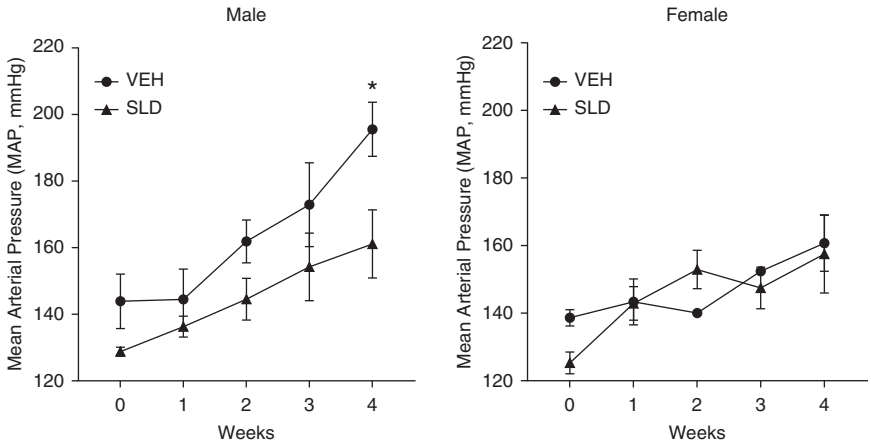

Figure 4. | **Mean arterial pressure (MAP) measured by telemetry increased over time as expected in all groups on 2% NaCl.** However, male offspring of SLD-treated dams exhibit a significantly attenuated rise as compared to that of VEH-fed dams (n=3–4 per group; *P<0.05).

NO (52), the renin-aldosterone-angiotensin system is unaffected. Another possibility is that AngII sensitivity programmed by exposure to preeclampsia is accomplished through an entirely different mechanism, such as catechol-O-methyl transferase deficiency, which has also been previously associated with preeclampsia (53,54). In fact, risk of preeclampsia has been associated with a low-activity catechol-O-methyl transferase genotype in the fetus (55).

A limitation of this study is that the data points generated from animals at 3 and 6 months of age consist of separate cohorts of animals, as euthanasia was necessary for collection

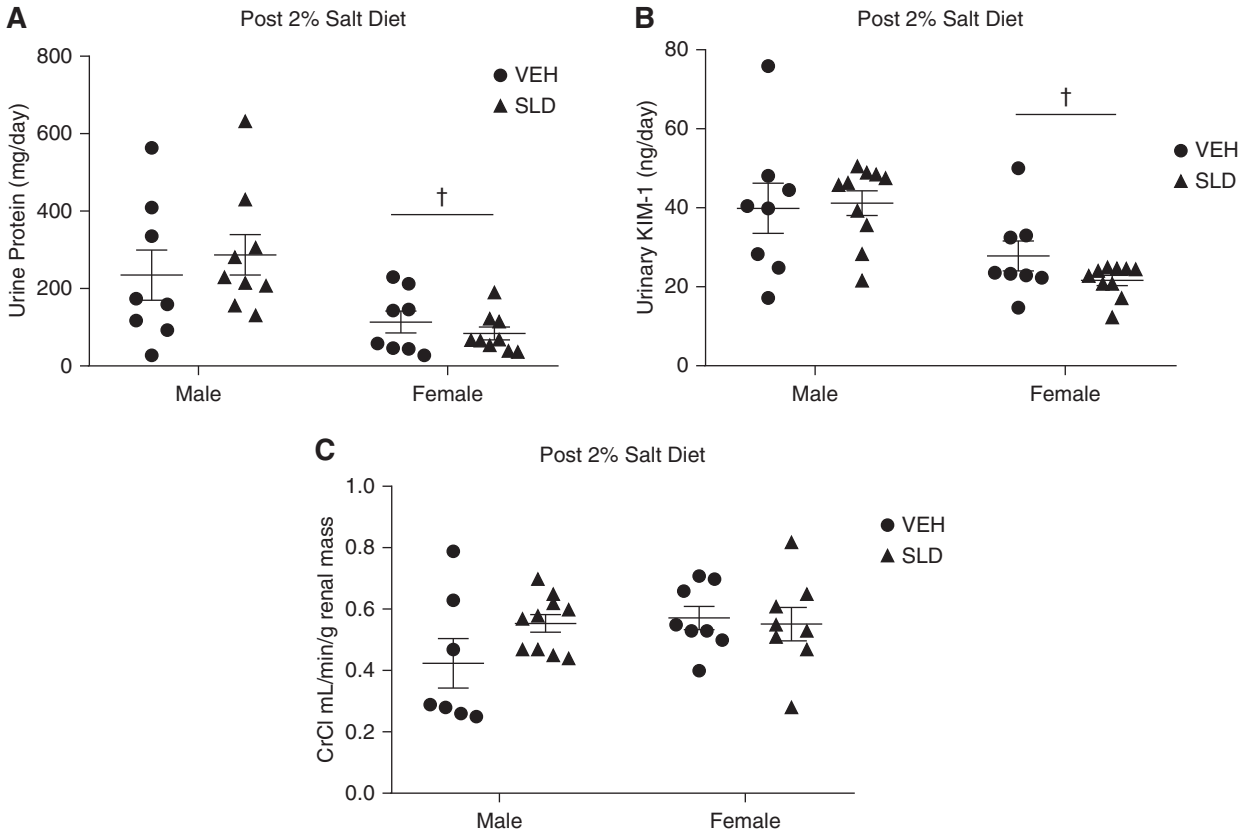

Figure 5. | **Female rats exhibit less renal injury after 2% salt diet; however, no significant differences are seen among maternal treatment groups.** (A) Female rats maintain lower proteinuria. (B) Female rats maintain less KIM-1 excretion. (C) No significant differences are seen in creatinine clearance (n=7–10 per group; $^{\dagger}P$<0.05 versus male).

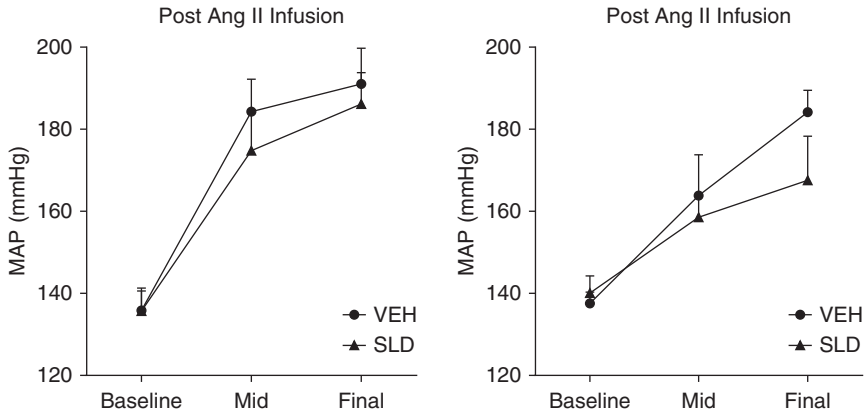

Figure 6. | MAP measured by telemetry of all groups increased in response to chronic angiotensin II (AngII) infusion. No significant differences were seen among treatment groups (n=5–6 per group).

of a complete data set. The number of data points required to achieve statistical power was such that the rats were also the product of several different cohort of dams, who were mated at different times throughout the year. The differing levels of stress on these dams, from small changes in temperature, humidity, or other conditions, cannot be underscored in the consideration of their effects on BP, and thus the fetal programming effects previously mentioned. These limitations may explain some of the variability seen in these studies, and a more rigorously controlled cohort of animals may further elucidate the mechanisms underlying the findings observed. Although we did not replicate previous findings indicating reduction of MAP in offspring of sildenafil-treated dams in early life (38), it is important to note that these were performed by two different methods (telemetry versus tail cuff). Tail-cuff readings are likely less

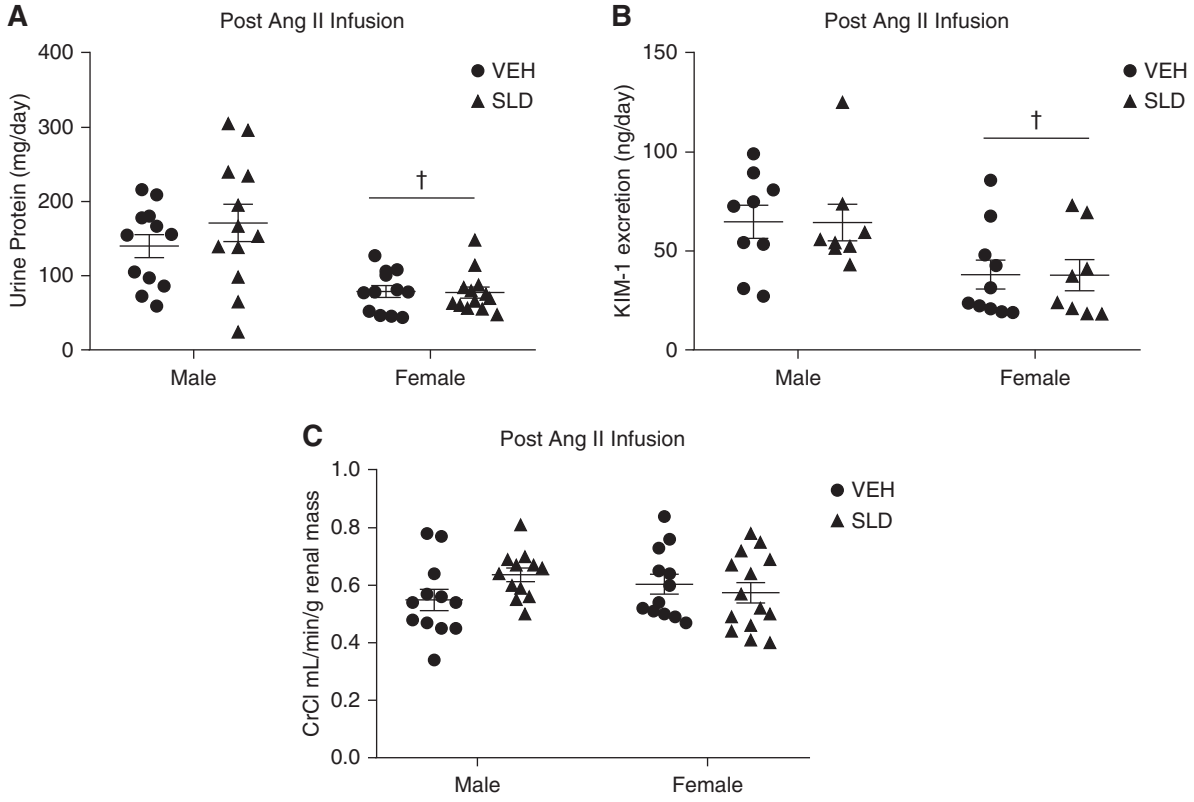

Figure 7. | No sex differences were observed in renal function or injury after chronic AngII infusion. Sex differences in renal injury as determined by (A) proteinuria and (B) KIM-1 excretion were maintained as previously described. (C) No differences are observed between treatment groups or sexes in renal function as measured by creatinine clearance (n=12–14 per group; $^{\dagger}P$<0.05 versus male).

sensitive than telemetry and the restraints necessary to tail-cuff measurement provide additional stress to the animals not seen in a telemetry setting, both of which may have contributed to the obscuration of the small differences previously observed. In this study, we used a moderately high-salt diet (2%) and a low-pressor dose of AngII to test sensitivity to additional stressors. However, it is possible that these regimens elicit a maximal response so that subtle effects of intrapartum sildenafil treatment could not be observed. Future studies will also include more detailed investigation of sodium transporter abundance/activity and renin-angiotensin-aldosterone system activation to parse out differential effects of sildenafil treatment on these two systems.

On the basis of these results, we suggest that therapies targeting the NO pathway during preeclampsia have the potential to reduce prenatal programming of salt-sensitive hypertension. However, our results do not support the hypothesis that sildenafil citrate reprograms the risk of hypertension and kidney disease in offspring of preeclamptic pregnancies.

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Author Contributions

M. Garrett was responsible for formal analysis, funding acquisition, methodology, and resources, and reviewed and edited the manuscript; A. Johnson contributed to data curation and formal analysis, and reviewed and edited the manuscript; J. Sasser conceptualized the study, was responsible for funding acquisition, methodology, project administration, and resources, and reviewed and edited the manuscript; H. Turbeville conceptualized the study, was responsible for data curation, formal analysis, funding acquisition, investigation, methodology, project administration, and validation, wrote the original draft, and reviewed and edited the manuscript.

Disclosures

All authors have nothing to disclose.

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Supplemental Material

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Supplemental Table 1. Gene targets and corresponding proteins quantified by targeted RNA sequencing as described in methods.

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Supplemental Tables

Table S1. Gene targets and corresponding proteins quantified by targeted RNA sequencing as described in methods.

GENE IDENTIFIER	PROTEIN
AGTR1A	Angiotensin II Receptor, type 1A
CAT	Catalase
COL3A1	Collagen Type III Alpha 1 chain
EDN1	Endothelin-1
EDNRA	Endothelin Receptor type A
ENDRB	Endothelin Receptor type B
GPX2	Glutathione Peroxidase 2
GSS	Glutathione Synthetase
HMOX1	Heme Oxygenase 1
HMOX2	Heme Oxygenase 2
HIF3A	Hypoxia-Inducible Factor 3 Alpha
IL10	Interleukin 10
IL17A	Interleukin 17A
IL6	Interleukin 6
HAVCR1	Hepatitis A Virus Cellular Receptor 1
NOX4	NADPH Oxidase 4
NPHS1	Nephrin
LCN2	Lipocalin 2
NOS2	Nitric Oxide Synthase 2, inducible nitric oxide synthase
NOS3	Nitric Oxide Synthase 3, endothelial nitric oxide synthase
PDE5A	Phosphodiesterase 5A
NPHS2	Podocin
PRKG2	cGMP-dependent protein kinase G
ATP6AP2	V-type proton ATPase
S100A4	S100 calcium binding protein A4
SOD1	Superoxide Dismutase 1
SOD2	Superoxide Dismutase 2
SOD3	Superoxide Dismutase 3
TGFB1	Tumor Growth Factor β1
TIMP1	TIMP metallopeptidase inhibitor 1
TNF	Tumor necrosis factor
VIM	Vimentin