Menopause: The Journal of The North American Menopause Society
Vol. 27, No. 12, pp. 1411-1419
DOI: 10.1097/GME.0000000000001675

ORIGINAL STUDY

Medroxyprogesterone opposes estradiol-induced renal damage in midlife ovariectomized Long Evans rats

Margaret A. Zimmerman, PhD,[1] Benard O. Ogola, PhD,[1] Mary M. Wilkinson, BS,[1] Bruna Visniauskas, PhD,[1] Carmen De Miguel, PhD,[2] Jill M. Daniel, PhD,[3,4] and Sarah H. Lindsey, PhD[1,3]

Abstract

Objective: Our laboratory previously published that long-term administration of estradiol (E_2) was detrimental to the kidneys of midlife ovariectomized Long Evans rats, contrasting clinical studies in showing that menopausal hormone therapy is associated with decreased albuminuria. However, it is unknown whether this renal benefit was due to estrogen and/or the combination with progestogen. Therefore, the objective of the current study was to determine the impact of medroxyprogesterone (MPA) on E_2-mediated renal damage using a rodent model.

Methods: Female Long Evans retired breeders underwent ovariectomy at 11 months of age and were treated for 40 days with subcutaneous E_2, E_2+MPA or vehicle at doses mimicking that of menopausal hormone therapy ($N = 5$-7 per group). Systolic blood pressure was measured along with indices of renal damage and function to investigate the impact of MPA on E_2-mediated renal outcomes. Renal estrogen receptor alpha and G protein-coupled estrogen receptor transcript copy numbers were measured in all treatment groups through droplet digital PCR.

Results: Middle-aged female Long Evans rats displayed spontaneous hypertension with similar systolic blood pressures and heart weights between groups. Even though blood pressure was comparable, E_2 reduced glomerular filtration rate and increased proteinuria indicating pressure-independent renal damage. Coadministration with MPA prevented E_2-induced glomerular filtration rate impairment and proteinuria by promoting renal hypertrophy and preventing renal interstitial fibrosis. Both E_2 and E_2+MPA reduced renal estrogen receptor alpha (ERα) and increased renal G protein-coupled estrogen receptor mRNA, but neither ERα nor ERß protein was different between groups.

Conclusion: MPA was protective against E_2-induced renal damage and dysfunction in middle-aged female Long Evans rats. Assessing the impact of hormone therapy on renal outcomes may be an important clinical factor when considering treatment options for postmenopausal women.

Key Words: Estradiol – Medroxyprogesterone – Menopause – Kidney – Long Evans rat.

Hormone therapy (HT) for healthy, symptomatic women is considered beneficial if they have no contraindications and are within 10 years of menopausal onset or younger than age 60 years.[1] HT improves quality of life for postmenopausal women by alleviating many of the physical symptoms of menopause, such as mood changes, sleep disruption, vasomotor instability, and vaginal atrophy.[2] However, the number of physicians prescribing HT for menopausal symptoms has drastically declined due to safety concerns raised from the Women's Health Initiative study which showed that HT increased the risk for breast cancer, coronary heart disease, stroke, and pulmonary embolism.[3] In response to the Women's Health Initiative study, HTs were shifted to a lower dose and shorter regimen, but the overall number of prescriptions never fully recovered as safety concerns still remain.[3]

Received April 6, 2020; revised and accepted August 3, 2020.

From the [1]Department of Pharmacology, Tulane University, New Orleans, LA; [2]Department of Medicine, Division of Nephrology, University of Alabama at Birmingham, Birmingham, AL; [3]Tulane Brain Institute, Tulane University, New Orleans, LA; and [4]Department of Psychology, Tulane University, New Orleans, LA.

Funding/Support: This work was supported by the National Institutes of Health - Heart, Lung, and Blood Institute [HL133619 to S.H.L. and HL145324 to C.D.M.], the National Institute on Aging [AG041374 to J.M.D.] and in part by the National Institute of General Medical Sciences [U54 GM104940], which funds the Louisiana Clinical and Translational Science Center. Services provided by Hypertension Core facility were supported by the National Institute of General Medical Sciences [P30 GM103337].

Portions of this work were presented and published for the American Heart Association Hypertension Scientific Sessions, held in New Orleans, LA at the New Orleans Marriott Hotel on September 7, 2019.

Financial disclosures/conflicts of interest: None reported.

Supplemental digital content is available for this article. Direct URL citations are provided in the HTML and PDF versions of this article on the journal's Website (www.menopause.org).

Address correspondence to: Margaret A. Zimmerman, PhD, Department of Pharmacology, Tulane University, 1430 Tulane Ave, mailbox 8683, New Orleans, LA 70112. E-mail: mzimmer5@tulane.edu

Systematic review and meta-analysis identifying the role of HT on kidney function suggests a potential benefit for reduced albuminuria, but there is still controversy due to discrepancies in the reported outcomes and study designs.[4] Although HT may provide benefits against menopause-related symptoms, a subset of women experience increased microalbuminuria and a reduction in glomerular filtration rate (GFR) in response to estrogen therapy.[5,6] Similarly, we previously published that chronic estrogen therapy worsened renal health in midlife ovariectomized (OVX) Long Evans rats,[7] and confirmed other reports showing increased proteinuria in castrated 14 month-old male Otsuka-Long-Evans-Tokushima Fatty rats.[8] Therefore, understanding the impact of HT on renal outcomes will remain a critical issue as the prevalence of menopause increases among a growing aging population.

HT for postmenopausal symptoms can include a combination of estrogen and progestogen, but women who have undergone a hysterectomy need only estrogen therapy. Medroxyprogesterone (MPA) is a progesterone derivative used to oppose uterine hyperplasia and reduce the risk of endometrial cancer. Our previously published results in the midlife female Long Evans rat mimics the response in postmenopausal women where estrogen therapy exhibits a negative effect on renal health.[5] Although other studies show that postmenopausal women using HT can experience a decrease in albuminuria,[4] it is unknown whether this benefit was due to estrogen alone and/or due to the progestogen combination. Therefore, the goal of the current study was to determine if coadministration of MPA antagonizes estrogen-mediated renal damage. The use of a rodent ''menopausal'' model will address a fundamental question about the impact of menopausal HT on the renal system and provide a foundation for future clinical studies.

METHODS

Animals

Female retired breeder Long Evans rats aged approximately 11 months were received from Envigo (Indianapolis, IN) and housed in a temperature-controlled Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium under a 12 hour light-dark cycle. Food and water were provided *ad libitum* and animals were maintained on Teklad Global Soy Protein-Free Extruded Rodent Diet 2020X from Envigo. All animals were OVX at 11.5 to 12 months of age and implanted with subcutaneous dorsal capsules or minipumps to deliver treatments for 40 days. Animals were matched by baseline blood pressure and assigned to receive either vehicle ($N=6$), estradiol (E_2, $N=7$) or E_2 plus MPA, ($N=6$). E_2 and vehicle treatments were administered using 5 mm silastic capsule (0.058 in. inner diameter and 0.077 in. outer diameter; Dow Corning, Midland, MI) containing either 25% 17β-E_2 (Sigma-Aldrich, St. Louis, MO) diluted with cholesterol as previously described.[7] Estradiol implants made with these exact dimensions maintain blood plasma E_2 levels in the physiological range and also match the E_2 levels achieved in postmenopausal women administered transdermal E_2.[9-11] Control rats received cholesterol-only implants, which we previously showed increase neither uterine weight nor serum E_2 levels and maintain a predominance of leukocytes in vaginal smears.[11,12] MPA was administered using 2ML4 osmotic minipumps at 1 mg/kg/day (ALZET, Cupertino, CA) and dissolved in 1:1 polyethylene glycol and dimethyl sulfoxide solution as described elsewhere.[13] This dose is slightly higher than the daily oral MPA dose for endometrial protection in postmenopausal women of approximately 0.15 mg/kg/day.[14] End of study wet weights of the uterus, kidney, and heart were obtained and normalized to body weight. Blood samples were collected via cardiac puncture and divided to collect plasma and serum samples. Serum and plasma samples were both centrifuged at 1000xg before removal of red blood cells. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the Tulane University Institutional Animal Care and Use Committee.

Blood pressure

Systolic blood pressure was recorded weekly using tail cuff plethysmography (CODA system, Kent Scientific, Torrington, CT). Animals were acclimated to the restraint for 2 days before baseline measurements. Animals were placed in restrainers and tails were warmed to 34°C. Blood pressures were taken over three days, taking the average of 15 readings per day excluding outliers (±2 SD). Final weekly pressures were averaged over the three consecutive days.

Urinary markers

Metabolic cages were used to collect the baseline and end of study urine. Samples were spun at 1000 x g for 10 minutes to remove particulates. Urinary protein concentration was measured via Bradford assay (Bio-Rad, Hercules, CA) using the standard bovine serum albumin and normalized against kilograms of body weight per day. Serum and urine creatinine were measured using the Jaffe Method and a 4:1 ratio of 1% Picric acid to 1N sodium hydroxide solution.[15] Creatinine values were measure at 492 nm with known concentrations of creatinine as assay standards. GFR was calculated as previously described.[7] All assays were performed in triplicate.

Histology

Renal morphology was assessed in formalin-fixed and paraffin-embedded 4-μm kidney sections mounted onto slides ($N=5$-6 per group). Slides were heated at 55°C for 30 minutes before being deparaffinized and hydrated. Trichrome Gomori One Step Aniline Blue Stain kit (Newcomer Supply, Middleton, WI) and NovaUltra Periodic Acid-Schiff Stain Kit (IHC World, Woodstock, MD) were used according to the manufacturer's directions. All histology measurements were analyzed using ten randomly selected fields of view at 20X magnification. For glomerulosclerosis, the average number of scored glomeruli was between 31 and 41 per slide. Glomerulosclerosis was assessed using a semiquantitative

score from 0 to 4 (0 = no fibrosis, 1 = 0%-25% of glomeruli fibrotic, 2 = 25%-50% of glomeruli fibrotic, 3 = 50%-75% of glomeruli fibrotic, and 4 = 75%-100% of glomeruli fibrotic). Damage to the renal proximal tubule brush border was graded using a scale of 0 to 3 for Periodic Acid-Schiff staining (0 = none, 1 = poor, 2 = moderate, and 3 = excellent), and the average score was obtained from ten randomly selected fields of view. A scoring system using four grades of severity in kidney damage (0 = absence, 1 = mild, 2 = moderate, and 3 = severe) was used to evaluate whole kidney tubulointerstitial fibrosis, protein cast formation, vasa recta hyalinosis, peritubular congestion, and proximal tubule dilation. All histological analyses were assessed and quantified by one independent investigator who was blinded to the treatment groups. Intraobserver variability ranged from 10% to 18%.

Droplet digital PCR

Estrogen receptor alpha (ERα) and G protein-coupled estrogen receptor (GPER) mRNA was measured from five randomly selected animals per group. ERß data is not provided because its expression was extremely low (<1 copy/ng RNA). Kidneys were collected and stored in RNAlater solution (ThermoFisher Scientific, Waltham, MA). Purified RNA was collected from harvested kidney tissue (50 mg) via the QIAGEN RNeasy Mini Kit (QIAGEN, Germantown, MD). Dual-labeled fluorescent probes for GPER (Assay ID: dRnoCPE5151056) and ERα (Assay ID: dRnoCPE5176827; Bio-Rad, Hercules, CA) were used with the Bio-Rad One-Step RT-ddPCR Advanced Kit, and droplets were analyzed in triplicate using the Bio-Rad QX200 system and QuantaSoft software, as previously described.[16]

Immunoblotting

Kidney cortex and uterus were homogenized and immunoblotted as previously described.[17] RIPA Lysis buffer containing protease and phosphatase inhibitors was added to 50 mg of tissue in a glass homogenizer. After obtaining a homogenous consistency, samples were centrifuged for 6 minutes at 12000 x g at 4°C and the supernatant was extracted. After protein determination using the BCA assay, 50 μg of kidney lysate and 20 μg uterine lysate were loaded onto a gel NuPage 10% Bis-Tris Gel (Invitrogen) and electrophoresis proceeded at 200 V. Gels were transferred to nitrocellulose membranes using the iBlot system (Invitrogen; Program 0, 7 min). After blocking with Odyssey Blocking Buffer (Li-Cor), membranes were incubated overnight at 4°C with monoclonal antibodies against ERα (Santa Cruz SC-8002, Lot G0717) and ERβ (Santa Cruz SC-373853, Lot G0117). GPER was not probed because antibodies failed validation in our hands. Odyssey IRDye 680RD Goat anti-Mouse IgG (Li-Cor 926-68070) was used as a secondary antibody and blots were imaged using the Odyssey system. Blots were reprobed with glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling 2118S; Lot 10) and secondary anti-rabbit as a loading control. Band densitometries were obtained using the ImageJ Gel Analyzer function.

Statistical analysis

Blood pressure, tissue weights, RNA, and urinary and serum markers were analyzed using a parametric one-way analysis of variance, followed by Tukey's multiple-comparisons test. Outliers were identified using the robust regression and outlier removal method, and data are presented as means ± standard error of the mean. Renal glomerulosclerosis and brush border scores were analyzed using a nonparametric Kruskal-Wallis test, followed by a Dunn's multiple-comparisons test when appropriate. Renal tubulointerstitial fibrosis, protein cast formation, vasa recta hyalinosis, peritubular congestion, and proximal tubule dilation were analyzed using Fisher-Freeman-Halton exact test, followed by Bonferroni correction when appropriate. Differences were considered statistically significant when $P < 0.05$. Analyses were performed using Prism version 6.0 (GraphPad Software, La Jolla, CA) and SPPS (IBM, v.22, Chicago, IL). All investigators were blinded to the treatment groups for the entirety of the study.

RESULTS

Baseline systolic blood pressure did not differ between groups (Veh: 144 ± 4; E_2: 143 ± 4 and E_2+MPA: 147 ± 4 mmHg; $P = 0.24$). There were no significant alterations from baseline blood pressure after 40 days of treatment or between groups (Veh: 135 ± 5; E_2: 143 ± 6 and E_2+MPA: 143 ± 5 mmHg; Figure 1A; $P = 0.55$). Body weights were comparable between groups before OVX (Veh: 348 ± 7; E_2: 345 ± 11 and E_2+MPA: 335 ± 10 g; $P = 0.44$) and after 40 days of treatment (Veh: 373 ± 13; E_2: 365 ± 19 and E_2+MPA: 339 ± 13 g; $P = 0.31$). Uterine weights were used to confirm treatment efficacy after OVX. As expected, E_2 significantly increased uterine weights compared with vehicle ($P < 0.001$). Unexpectedly, coadministration with MPA enhanced the impact of E_2 on uterine weight (Figure 1B; $P = 0.003$). E_2 therapy did not alter kidney weights from vehicle unless it was coadministered with MPA (Figure 1C; $P = 0.002$). There were no alterations in heart weights between treatment groups (Figure 1D).

E_2 therapy for 40 days significantly increased proteinuria compared with vehicle (Figure 2A; $P = 0.028$). The E_2-induced proteinuria was absent during MPA coadministration ($P = 0.100$). E_2 therapy did not alter urinary creatinine (Figure 2B; $P = 0.124$) but significantly increased serum levels (Figure 2C; $P = 0.003$) compared with vehicle. In contrast, E_2+MPA significantly increased urinary creatinine ($P = 0.046$) but reduced serum levels ($P = 0.032$) compared with E_2 therapy. E_2 therapy significantly reduced GFR ($P < 0.001$ vs vehicle), but coadministration of MPA with E_2 reversed the reduction (Figure 2D; $P = 0.139$ vs vehicle).

Glomerulosclerosis was mild and not significantly different between treatment groups (Table 1; $P = 0.86$). Renal brush border scores were not significantly different between groups ($P = 0.151$), with most sections displaying poor to moderate staining. Additional pathological changes in the kidneys due to E_2 and E_2+MPA were observed but not statistically

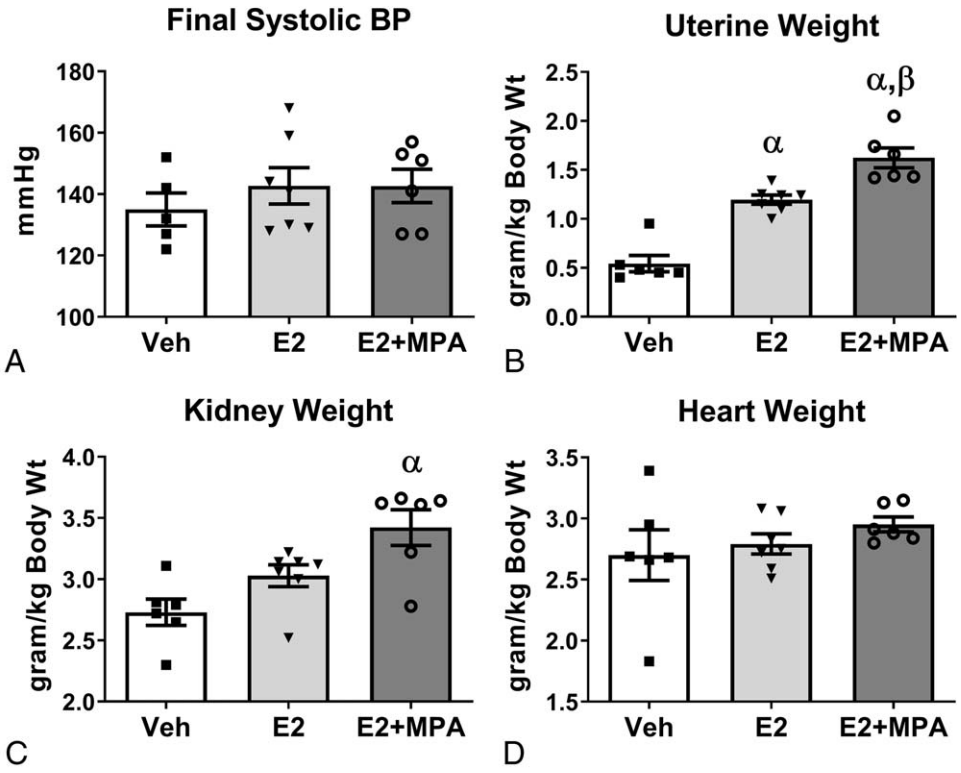

FIG. 1. Animals received 40 d of vehicle (Veh), estradiol (E_2) or estradiol + medroxyprogesterone (E_2+MPA) immediately following ovariectomy. **(A)** Final systolic blood pressure (BP), **(B)** uterine weight, **(C)** kidney weight, and **(D)** heart weight normalized to body weight. Values are means ± SEM, $N = 6$-7 per group; One-way ANOVA, $^{\alpha}P < 0.05$ vs. Veh, $^{\beta}P < 0.05$ vs E_2. ANOVA, analysis of variance; SEM, standard error of the mean.

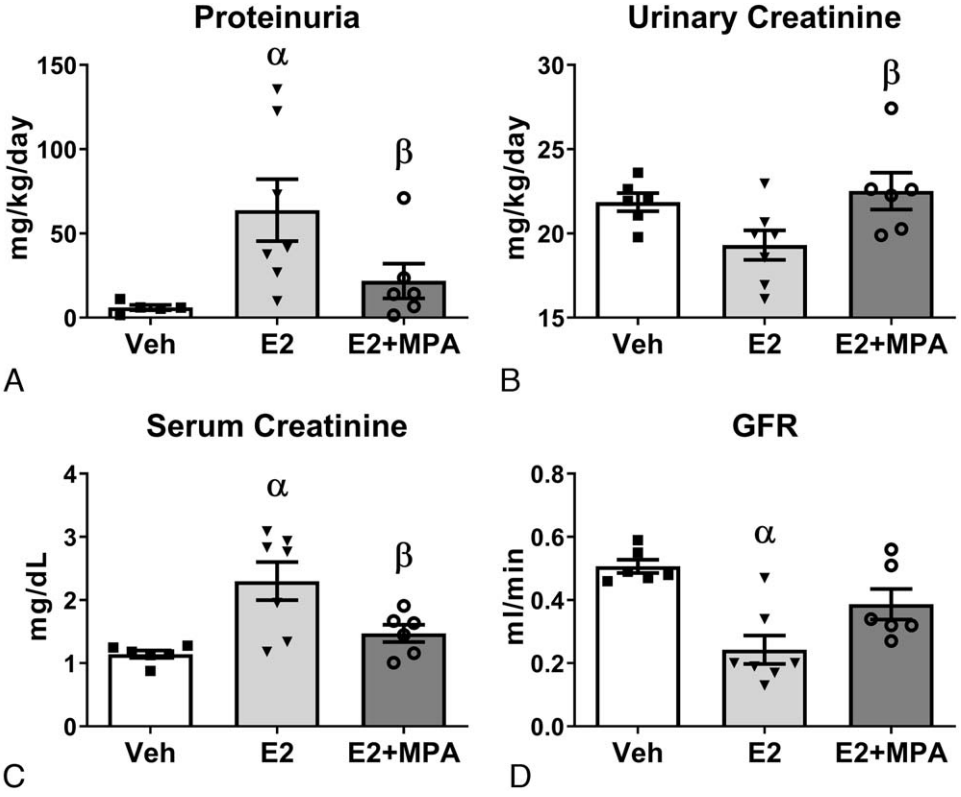

FIG. 2. Post-treatment **(A)** 24 h proteinuria, **(B)** urinary creatinine, **(C)** serum creatinine, and **(D)** glomerular filtration rate (GFR). Values are means ± SEM, N=6-7 per group; One-way ANOVA, $^{\alpha}P < 0.05$ vs Veh, $^{\beta}P < 0.05$ vs E_2. ANOVA, analysis of variance; SEM, standard error of the mean.

TABLE 1. *Renal pathological changes assessed using index scores for glomerulosclerosis, brush border staining, protein cast formation, vasa recta hyalinosis, peritubular congestion, proximal tubule dilation, and interstitial fibrosis. Chi-square analysis results are reported in the text.*

	Veh ($n=5$)	E_2 ($n=6$)	E_2+MPA ($n=6$)
Glomerulosclerosis			
no fibrosis	0 (0%)	0 (0%)	0 (0%)
0%-25% fibrotic	3 (60%)	1 (17%)	3 (50%)
25%-50% fibrotic	2 (40%)	5 (83%)	2 (33%)
50%-75% fibrotic	0 (0%)	0 (0%)	1 (17%)
75%-100% fibrotic	0 (0%)	0 (0%)	0 (0%)
Brush border			
None	0 (0%)	0 (0%)	0 (0%)
Poor	5 (100%)	3 (50%)	5 (83%)
Moderate	0 (0%)	1 (17%)	1 (17%)
Excellent	0 (0%)	2 (33%)	0 (0%)
Protein casts			
Absence	4 (80%)	1 (16%)	3 (50%)
Mild	1 (20%)	3 (52%)	1 (16%)
Moderate	0 (0%)	1 (16%)	0 (0%)
Severe	0 (0%)	1 (16%)	2 (34%)
Vasa recta hyalinosis			
Absence	4 (80%)	1 (17%)	5 (83%)
Mild	1 (20%)	4 (66%)	1 (17%)
Moderate	0 (0%)	0 (0%)	0 (0%)
Severe	0 (0%)	1 (17%)	0 (0%)
Peritubular congestion			
Absence	4 (80%)	4 (66%)	2 (34%)
Mild	0 (0%)	2 (34%)	4 (66%)
Moderate	0 (0%)	0 (0%)	0 (0%)
Severe	1 (20%)	0 (0%)	0 (0%)
Proximal tubule dilation			
Absence	4 (80%)	4 (66%)	5 (83%)
Mild	1 (20%)	2 (34%)	1 (17%)
Moderate	0 (0%)	0 (0%)	0 (0%)
Severe	0 (0%)	0 (0%)	0 (0%)
Interstitial fibrosis			
Absence	5 (100%)	1 (17%)	4 (66%)
Mild	0 (0%)	2 (33%)	1 (17%)
Moderate	0 (0%)	3 (50%)	0 (0%)
Severe	0 (0%)	0 (0%)	1 (17%)

significant. Renal protein cast formation was present in 1 out of 5 animals in the vehicle group compared with 5 out of 6 animals in the E_2 group and 3 out of 6 in the E_2+MPA group ($P=0.213$). Vasa recta hyalinosis was present in 1 out of 5 animals in the vehicle group compared with 4 out of 6 in the E_2 group and 1 out of 6 in the E_2+MPA group ($P=0.382$). Peritubular congestion and proximal tubule dilation were similar between groups ($P=0.127$ and $P=1.000$, respectively). The degree of renal fibrosis was significantly different between groups (Figure 3, $P=0.014$). Specifically, fibrosis was undetectable in 100% of the vehicle group compared with only 16.7% of the E_2 group and 66.7% in rats that were coadministered MPA.

We recently published that ERα is the predominant estrogen receptor in the kidney followed by GPER.[18] Moreover, ERα and GPER are suggested to play a renoprotective role in rodents.[17,19] Therefore, we evaluated whether alterations in renal estrogen receptor RNA or protein levels correlated with the adverse renal outcomes observed in our animal model. Results in the current study showed that renal ERα in both the E_2 and E_2+MPA treatment groups were comparable to each other but significantly lower than the vehicle group (Figure 4A; $P=0.018$). In contrast, renal GPER levels were comparable between E_2 and E_2+MPA groups but significantly greater than the vehicle group (Figure 4B; $P=0.020$). Renal ERß was below the level of detection, similar to our previous findings in Sprague-Dawley rats.[18] Immunoblotting for ERα and ERß showed no differences between treatment groups (Figure 4C and 4D). We did not probe for GPER because no commercially available antibodies were able to pass validation studies using knockout tissue. ERα and ERß protein expression was highly correlated (Pearson $r=0.97$, $P<0.0001$, $n=12$), and there was a negative but nonsignificant correlation between ERα protein and mRNA (Pearson $r=$-0.35, $P=0.39$, $n=8$). Full length blots are provided in Supplemental Table 1, http://links.lww.com/MENO/A670.

DISCUSSION

The novel finding from the current study was that coadministration of MPA blunted E_2-induced renal damage and dysfunction in midlife OVX Long Evans rats. This study confirmed our previously published results that chronic E_2 administration reduced GFR and increased proteinuria, whereas new data shows that these effects are prevented by

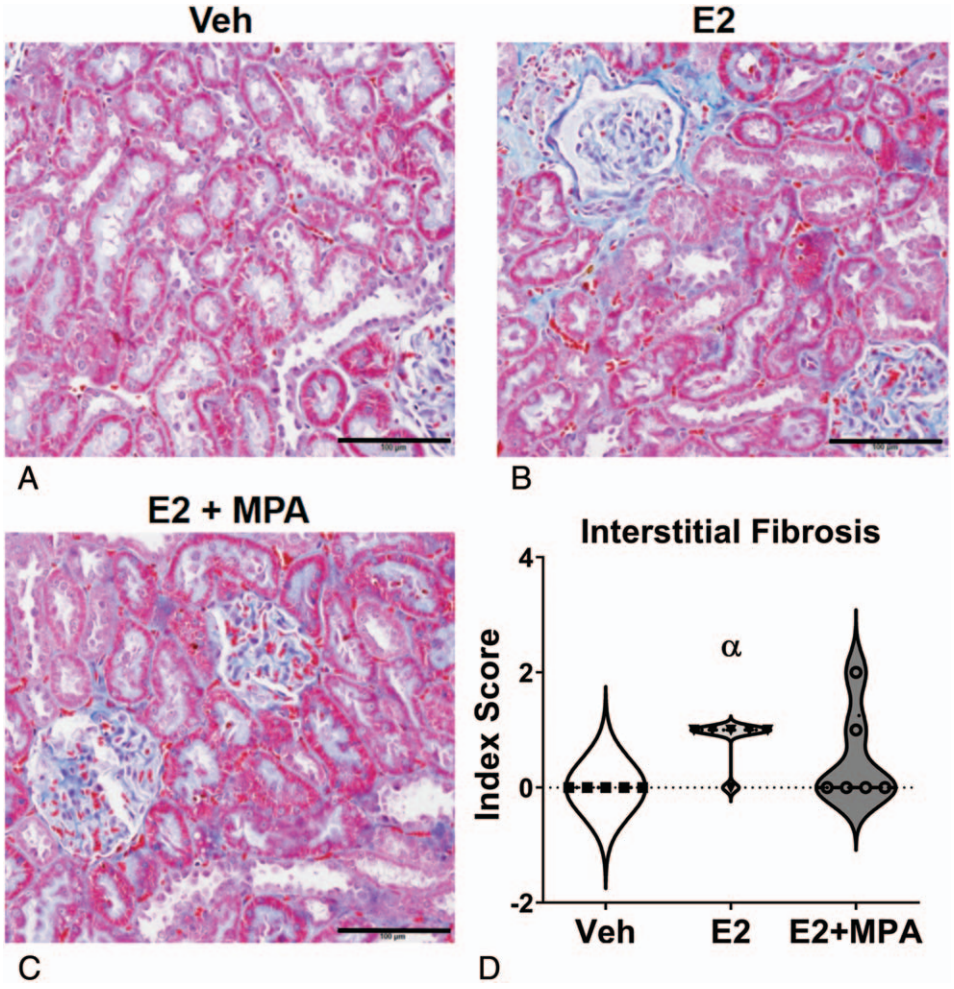

FIG. 3. Representative images of renal tubulointerstitial fibrosis assessed in trichrome stained kidney sections at 20X magnification (100 μm scale bar) following 40 d of **(A)** vehicle (Veh), **(B)** estradiol (E_2), or **(C)** estradiol + medroxyprogesterone (E_2+MPA). **(D)** Violin plot showing the data distribution using a scoring system to assess four grades of severity (0 = absence, 1 = mild, 2 = moderate, and 3 = severe). N=5-6 per group; Fisher exact test, $^{\alpha}P = 0.014$ vs Veh.

MPA coadministration. In addition, our results support clinical data indicating that estrogen therapies may have worse renal outcomes when not combined with a progestogen.[5]

The physiological effects of estrogen are diverse and dependent on the target tissue. In the kidney, female sex hormones are assumed to be protective since renal disease progresses more slowly in women versus aged-match men before the onset of menopause.[20,21] Meta-analysis reveals mixed results when evaluating the impact of estrogen therapy on albuminuria and proteinuria.[4] In a small prospective study, estrogen plus norgestrel reduced proteinuria and improved creatinine clearance in postmenopausal women with hypertension and type 2 diabetes.[22] The cross-sectional Rancho Bernardo Study found better GFR in hormone users, especially with long-term estrogen use.[23] Other studies show negative renal outcomes, including a large case-controlled study using data from the Prevention of Renal and Vascular End Stage Disease cohort, which found that menopausal HT is associated with increased microalbuminuria.[24] An observational study of premenopausal women with or without diabetes also found a positive association between oral contraceptive use and macroalbuminuria.[25] After correcting for age, diabetes, comorbidities, and baseline eGFR, the decline in eGFR was significantly greater with HT versus nonusers.[5] This confusing mixture of positive, negative, and neutral results has led to a lack of consensus on the effects of HT on renal outcomes.

Clinical studies assessing a potential renoprotective mechanism using progestogens in combination with estrogen add to the difficulty because not all progesterone derivatives are equal. For example, Norgestrel and gestodene stimulate the proliferation of breast cancer cells, while MPA does not impact cell growth.[26] Unexpected adverse cardiovascular effects in response to HT may also be amplified by natural aging. Estrogen administration in aged female spontaneously hypertensive rats is less effective at reducing cardiac hypertropy when compared to young animals,[27] indicating that the process of aging disrupts the protective effects. Therefore, discrepancies in clinical studies assessing renal health

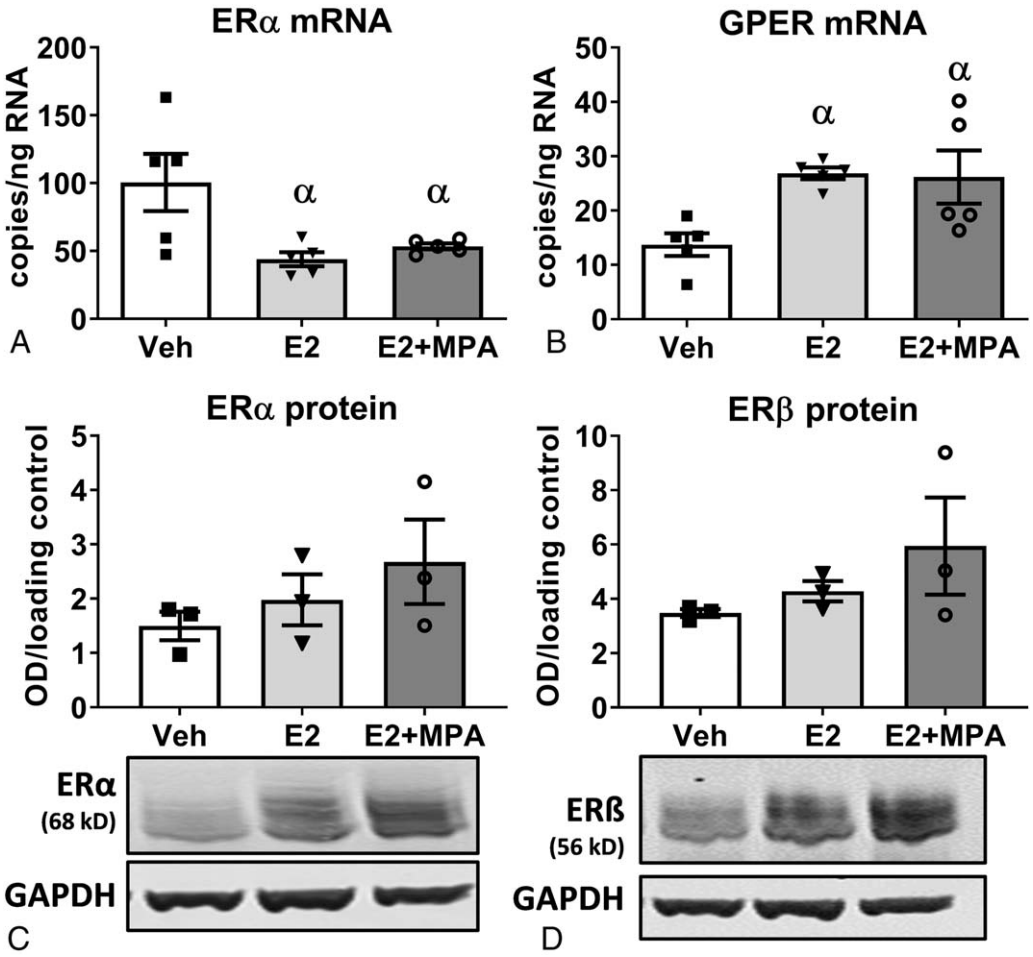

FIG. 4. **(A)** Renal ERα and **(B)** renal G protein-coupled estrogen receptor transcript copies per ng RNA after 40 days of vehicle (Veh), estradiol (E_2) or estradiol + medroxyprogesterone (E_2+MPA) treatment. Values are means ± SEM, $N = 5$-6 per group; One-way ANOVA, $^{\alpha}P < 0.05$ vs. Veh. **(C)** Renal ERα and **(D)** renal ERß protein expression was not different between groups. Values are means ± SEM, $N = 3$ per group; One-way ANOVA, $P > 0.05$. ANOVA, analysis of variance; SEM, standard error of the mean.

outcomes may also occur due to differences in hormone formulation, route of administration, treatment duration, and timing of menopause onset versus HT initiation.

The current study showed that middle-aged female Long Evans rats display spontaneous hypertension, with the final systolic blood pressure and heart weights consistent between groups following the 40-day treatment. Therefore, E_2-reduced GFR and increased proteinuria independent of blood pressure alterations. Coadministration with MPA prevented E_2-induced renal dysfunction through compensatory renal hypertrophy, a mechanism that enhances the functional capacity of the residual nephrons to maintain normal renal function. Tubulointerstitial fibrosis promotes renal disease progression due to the excessive accumulation of extracellular matrix in the renal interstitium,[28] and our results suggest that MPA reduced E_2-mediated renal damage by preventing kidney fibrosis. Progestogens are designed to stimulate progesterone receptors but can also influence additional steroid receptors, such as mineralocorticoid, glucocorticoid and androgen-receptors.[29-31] Therefore, modulation of nonprogesterone receptors may play a role in the MPA response to protect the kidney against E_2-mediated damage. Further studies will need to determine the molecular mechanism by which MPA influences the extracellular matrix, primarily its role in renal interstitial collagen formation.

Depletion of sex hormones with OVX resulted in reduced uterine weight, and treatment efficacy for E_2 was confirmed by an increase in uterine weight compared with vehicle. Our adverse renal effects are not suspected to be the result of high E_2 levels since we previously reported serum E_2 levels to be approximately 15 pg/mL,[7] which is lower than the plasma E_2 levels of 30 pg/mL measured in intact female Long Evans rats.[32] Progestogens are commonly used to antagonize estrogen-induced growth in uterine tissue since it reduces and inhibits the replenishment of uterine estrogen receptors, and therefore, leads to reduced sensitivity to further estrogen stimuli and depressed uterine weight.[33] Therefore, we expected to see attenuation of uterine weight in response to MPA as reported elsewhere.[34-36] However, MPA-induced uterotrophic effects[37,38] or lack of antagonism of estrogen-induced uterotrophy[34,39-41] are also reported. These differences are independent of dosing, as the same dose of MPA produces opposite results in similar animal

models.[35,38] Like most other steroids, progesterone binds to a membrane receptor in addition to its nuclear targets.[42] Progesterone receptor membrane component (PGRMC) 1 and PGRMC2 are both expressed in rat ovary[43,44] as well as in mouse and human uterine tissue.[45] Some actions of MPA can be inhibited with PGRMC1 silencing,[46] and PGRMC knockout mice have abnormal uteri with increased glandular content.[47] A role for PGRMC in either uterine or kidney hypertrophy, however, is yet to be established.

Sex hormones regulate estrogen receptor expression,[48,49] but the function of these receptors in the kidney is not well-defined. Female global ERα knockout mice display greater proteinuria and glomerular damage compared with the wild-type,[19] whereas, renal mesangial ERα expression is inversely correlated with glomerulosclerosis.[50] These studies indicate that ERα plays a renoprotective role and may impact renal health outcomes. The current study showed that 40 days of E_2 treatment in OVX Long Evans rats reduced renal ERα mRNA but not protein, confirming a similar effect after 120 days of E_2 therapy in OVX Sprague Dawley rats,[49] however this transcriptional regulation did not correlate with ERα protein. Additional studies are needed to determine the feedback loop for ERα regulation in the kidney. Progesterone modulates ERα expression in uterine tissue during estrogen administration,[51] but we did not find a difference in renal ERα levels between E_2 and E_2+MPA groups. ERα levels in the uterus are necessary for appropriate proliferation and thickening of the endometrium and are approximately two-fold higher than in the kidney of Sprague Dawley rats.[18] Therefore, the ability of progesterone to regulate ERα expression may be absent in nonreproductive tissues such as the kidney.[52,53]

The differing regulation of estrogen receptors in response to E_2 may protect estrogen-sensitive tissues from over responding. E_2 treatment in Michigan Cancer Foundation-7 breast cancer cells causes a rapid reduction in both ERα and GPER, but GPER levels recover after 96 hours.[54] GPER was assessed in the current study since it is the second predominant renal estrogen receptor in rats and protective in cardiovascular and renal tissues.[18,55] We found an inverse relationship between renal ERα and GPER transcript levels, where ERα was downregulated and GPER was upregulated compared with controls. However, there was no difference between the E_2 and E_2+MPA groups despite GFR and proteinuria being worse in the E_2 group. Therefore, our study indicates that the kidneys are estrogen-sensitive, but the adverse E_2-mediated renal outcomes may be independent of alterations in expression.

Potential clinical value

There is a lack of consensus on the effects of HT on renal outcomes because clinical trials are limited and inconsistent. A survey of nephrologists shows that 43% are uncertain about the impact of menopausal HT in women with chronic kidney disease,[56] indicating that a standard of care for HT use in healthy women and in patients with reduced kidney function is needed. The current study used an aging rodent ''menopausal'' model to show that co-administration of MPA prevented the adverse renal outcomes associated with E_2 therapy. These findings indicate that clinical administration of HTs containing MPA may offer protection for patients that are susceptible to renal damage during estrogen use. Future studies will need to determine the molecular mechanism by which MPA protects renal health and whether the protective benefits extend to other progestogen formulas.

CONCLUSIONS

The present study demonstrated that chronic estrogen therapy had a different impact on renal health when not administered with a progestogen. Specifically, MPA attenuated the negative renal effects of E_2 in midlife OVX Long Evans rats, independent of alterations in blood pressure. Furthermore, our results show that chronic exposure to E_2 or E_2+MPA therapy impacts renal estrogen receptor RNA levels, where ERα was downregulated and GPER was upregulated. Therefore, assessing renal markers of injury in postmenopausal women on HT may be clinically important and may help in decisions about dosage and discontinuation.

REFERENCES

1. The 2017 hormone therapy position statement of The North American Menopause Society. *Menopause* 2017;24:728-753.
2. Gracia CR, Freeman EW. Onset of the menopause transition: the earliest signs and symptoms. *Obstet Gynecol Clin North Am* 2018;45:585-597.
3. Ettinger B, Wang SM, Leslie RS, et al. Evolution of postmenopausal hormone therapy between 2002 and 2009. *Menopause* 2018;25:1306-1312.
4. Kattah AG, Suarez MLG, Milic N, et al. Hormone therapy and urine protein excretion: a multiracial cohort study, systematic review, and meta-analysis. *Menopause* 2018;25:625-634.
5. Ahmed SB, Culleton BF, Tonelli M, et al. Oral estrogen therapy in postmenopausal women is associated with loss of kidney function. *Kidney Int* 2008;74:370-376.
6. Agarwal M, Selvan V, Freedman BI, Liu Y, Wagenknecht LE. The relationship between albuminuria and hormone therapy in postmenopausal women. *Am J Kidney Dis* 2005;45:1019-1025.
7. Zimmerman MA, Hutson DD, Trimmer EH, et al. Long- but not short-term estradiol treatment induces renal damage in midlife ovariectomized Long-Evans rats. *Am J Physiol Renal Physiol* 2017;312:F305-F311.
8. Tomiyoshi Y, Sakemi T, Aoki S, Miyazono M. Different effects of castration and estrogen administration on glomerular injury in spontaneously hyperglycemic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Nephron* 2002;92:860-867.
9. Andersson TL, Stehle B, Davidsson B, Hoglund P. Drug concentration effect relationship of estradiol from two matrix transdermal delivery systems: Menorest and Climara. *Maturitas* 2000;35:245-252.
10. Gordon SF. Clinical experience with a seven-day estradiol transdermal system for estrogen replacement therapy. *Am J Obstet Gynecol* 1995;173:998-1004.
11. Bohacek J, Daniel JM. Increased daily handling of ovariectomized rats enhances performance on a radial-maze task and obscures effects of estradiol replacement. *Hormones Behav* 2007;52:237-243.
12. Bohacek J, Daniel JM. The beneficial effects of estradiol on attentional processes are dependent on timing of treatment initiation following ovariectomy in middle-aged rats. *Psychoneuroendocrinology* 2010;35:694-705.
13. Braden BB, Talboom JS, Crain ID, et al. Medroxyprogesterone acetate impairs memory and alters the GABAergic system in aged surgically menopausal rats. *Neurobiol Learn Mem* 2010;93:444-453.
14. Stanczyk FZ, Hapgood JP, Winer S, Mishell DR Jr. Progestogens used in postmenopausal hormone therapy: differences in their pharmacological properties, intracellular actions, and clinical effects. *Endocrine Rev* 2013;34:171-208.

15. Toora BD, Rajagopal G. Measurement of creatinine by Jaffe's reaction–determination of concentration of sodium hydroxide required for maximum color development in standard, urine and protein free filtrate of serum. *Indian J Exp Biol* 2002;40:352-354.
16. Zimmerman MA, Hutson DD, Mauvais-Jarvis F, Lindsey SH. Bazedoxifene-induced vasodilation and inhibition of vasoconstriction is significantly greater than estradiol. *Menopause* 2019;26:172-181.
17. Lindsey SH, Yamaleyeva LM, Brosnihan KB, Gallagher PE, Chappell MC. Estrogen receptor GPR30 reduces oxidative stress and proteinuria in the salt-sensitive female mRen2.Lewis rat. *Hypertension* 2011;58:665-671.
18. Hutson DD, Gurrala R, Ogola BO, et al. Estrogen receptor profiles across tissues from male and female *Rattus norvegicus*. *Biol Sex Differ* 2019;10:4.
19. Elliot SJ, Berho M, Korach K, et al. Gender-specific effects of endogenous testosterone: female alpha-estrogen receptor-deficient C57Bl/6J mice develop glomerulosclerosis. *Kidney Int* 2007;72:464-472.
20. Carrero JJ. Gender differences in chronic kidney disease: underpinnings and therapeutic implications. *Kidney Blood Press Res* 2010;33:383-392.
21. Neugarten J, Acharya A, Silbiger SR. Effect of gender on the progression of nondiabetic renal disease: a meta-analysis. *J Am Soc Nephrol* 2000;11:319-329.
22. Szekacs B, Vajo Z, Varbiro S, et al. Postmenopausal hormone replacement improves proteinuria and impaired creatinine clearance in type 2 diabetes mellitus and hypertension. *BJOG* 2000;107:1017-1021.
23. Fung MM, Poddar S, Bettencourt R, Jassal SK, Barrett-Connor E. A cross-sectional and 10-year prospective study of postmenopausal estrogen therapy and blood pressure, renal function, and albuminuria: the Rancho Bernardo Study. *Menopause* 2011;18:629-637.
24. Monster TB, Janssen WM, de Jong PE, de Jong-van den Berg LT. Prevention of R, vascular end stage disease study G. Oral contraceptive use and hormone replacement therapy are associated with microalbuminuria. *Arch Intern Med* 2001;161:2000-2005.
25. Ahmed SB, Hovind P, Parving HH, et al. Oral contraceptives, angiotensin-dependent renal vasoconstriction, and risk of diabetic nephropathy. *Diabetes Care* 2005;28:1988-1994.
26. Catherino WH, Jeng MH, Jordan VC. Norgestrel and gestodene stimulate breast cancer cell growth through an oestrogen receptor mediated mechanism. *Br J Cancer* 1993;67:945-952.
27. Jazbutyte V, Hu K, Kruchten P, et al. Aging reduces the efficacy of estrogen substitution to attenuate cardiac hypertrophy in female spontaneously hypertensive rats. *Hypertension* 2006;48:579-586.
28. Iwano M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. *Curr Opin Nephrol Hypertens* 2004;13:279-284.
29. Africander D, Verhoog N, Hapgood JP. Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. *Steroids* 2011;76:636-652.
30. Myles K, Funder JW. Progesterone binding to mineralocorticoid receptors: in vitro and in vivo studies. *Am J Physiol* 1996;270:E601-E607.
31. Thomas CP, Liu KZ, Vats HS. Medroxyprogesterone acetate binds the glucocorticoid receptor to stimulate alpha-ENaC and sgk1 expression in renal collecting duct epithelia. *Am J Physiol Renal physiol* 2006;290: F306-F312.
32. Clark JT, Chakraborty-Chatterjee M, Hamblin M, Wyss JM, Fentie IH. Estrogen depletion differentially affects blood pressure depending on age in Long-Evans rats. *Endocrine* 2004;25:173-186.
33. Hsueh AJ, Peck EJ Jr, clark JH. Progesterone antagonism of the oestrogen receptor and oestrogen-induced uterine growth. *Nature* 1975;254:337-339.
34. Di Carlo F, Racca S, Conti G, et al. Effects of long-term administration of high doses of medroxyprogesterone acetate on hormone receptors and target organs in the female rat. *J Endocrinol* 1984;103:287-293.
35. Dinh H, Nathan L. Medroxyprogesterone acetate does not antagonize estrogen-induced increases in endothelium-dependent vasodilation: potential clinical implications. *Fertil Steril* 2002;78:122-127.
36. McNeill AM, Zhang C, Stanczyk FZ, Duckles SP, Krause DN. Estrogen increases endothelial nitric oxide synthase via estrogen receptors in rat cerebral blood vessels: effect preserved after concurrent treatment with medroxyprogesterone acetate or progesterone. *Stroke* 2002;33:1685-1691.
37. Arias-Loza PA, Hu K, Schafer A, et al. Medroxyprogesterone acetate but not drospirenone ablates the protective function of 17 beta-estradiol in aldosterone salt-treated rats. *Hypertension* 2006;48:994-1001.
38. Braden BB, Garcia AN, Mennenga SE, et al. Cognitive-impairing effects of medroxyprogesterone acetate in the rat: independent and interactive effects across time. *Psychopharmacology* 2011;218:405-418.
39. Chisholm NC, Juraska JM. Effects of long-term treatment with estrogen and medroxyprogesterone acetate on synapse number in the medial prefrontal cortex of aged female rats. *Menopause* 2012;19:804-811.
40. Chisholm NC, Packard AR, Koss WA, Juraska JM. The effects of long-term treatment with estradiol and medroxyprogesterone acetate on tyrosine hydroxylase fibers and neuron number in the medial prefrontal cortex of aged female rats. *Endocrinology* 2012;153:4874-4882.
41. Jeanes HL, Wanikiat P, Sharif I, Gray GA. Medroxyprogesterone acetate inhibits the cardioprotective effect of estrogen in experimental ischemia-reperfusion injury. *Menopause* 2006;13:80-86.
42. Falkenstein E, Meyer C, Eisen C, Scriba PC, Wehling M. Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells. *Biochem Biophys Res Commun* 1996;229:86-89.
43. Peluso JJ, Pappalardo A, Losel R, Wehling M. Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action. *Endocrinology* 2006;147: 3133-3140.
44. Griffin D, Liu X, Pru C, Pru JK, Peluso JJ. Expression of progesterone receptor membrane component-2 within the immature rat ovary and its role in regulating mitosis and apoptosis of spontaneously immortalized granulosa cells. *Biol Reprod* 2014;91:36.
45. Zhang L, Kanda Y, Roberts DJ, et al. Expression of progesterone receptor membrane component 1 and its partner serpine 1 mRNA binding protein in uterine and placental tissues of the mouse and human. *Mol Cell Endocrinol* 2008;287:81-89.
46. Allen TK, Feng L, Grotegut CA, Murtha AP. Progesterone receptor membrane component 1 as the mediator of the inhibitory effect of progestins on cytokine-induced matrix metalloproteinase 9 activity in vitro. *Reprod Sci* 2014;21:260-268.
47. Clark NC, Pru CA, Yee SP, Lydon JP, Peluso JJ, Pru JK. Conditional ablation of progesterone receptor membrane component 2 causes female premature reproductive senescence. *Endocrinology* 2017;158:640-651.
48. Esqueda ME, Craig T, Hinojosa-Laborde C. Effect of ovariectomy on renal estrogen receptor-alpha and estrogen receptor-beta in young salt-sensitive and -resistant rats. *Hypertension* 2007;50:768-772.
49. Mohamed MK, Abdel-Rahman AA. Effect of long-term ovariectomy and estrogen replacement on the expression of estrogen receptor gene in female rats. *Eur J Endocrinol* 2000;142:307-314.
50. Potier M, Karl M, Zheng F, Elliot SJ, Striker GE, Striker LJ. Estrogen-related abnormalities in glomerulosclerosis-prone mice: reduced mesangial cell estrogen receptor expression and prosclerotic response to estrogens. *Am J Pathol* 2002;160:1877-1885.
51. DeMayo FJ, Zhao B, Takamoto N, Tsai SY. Mechanisms of action of estrogen and progesterone. *Ann N Y Acad Sci* 2002;955:48-59.
52. Arias-Loza PA, Muehlfelder M, Elmore SA, et al. Differential effects of 17beta-estradiol and of synthetic progestins on aldosterone-salt-induced kidney disease. *Toxicol Pathol* 2009;37:969-982.
53. Vereide AB, Kaino T, Sager G, Arnes M, Orbo A. Effect of levonorgestrel IUD and oral medroxyprogesterone acetate on glandular and stromal progesterone receptors (PRA and PRB), and estrogen receptors (ER-alpha and ER-beta) in human endometrial hyperplasia. *Gynecol Oncol* 2006;101:214-223.
54. Ariazi EA, Brailoiu E, Yerrum S, et al. The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* 2010;70:1184-1194.
55. Zimmerman MA, Budish RA, Kashyap S, Lindsey SH. GPER-novel membrane oestrogen receptor. *Clin Sci* 2016;130:1005-1016.
56. Ramesh S, James MT, Holroyd-Leduc JM, et al. Sex hormone status in women with chronic kidney disease: survey of nephrologists' and renal allied health care providers' perceptions. *Can J Kidney Health Dis* 2017;4:2054358117734534.