Effect of Ovariectomy on Renal Estrogen Receptor-α and Estrogen Receptor-β in Young Salt-Sensitive and -Resistant Rats

Ma. Eugenia Davila Esqueda, Teresa Craig, Carmen Hinojosa-Laborde

Abstract—This study evaluated the effect of ovariectomy on renal estrogen receptor (ER)-α and ERβ expression in young female Dahl salt-sensitive and salt-resistant rats. Our hypothesis was that estrogen depletion results in an imbalance in ERα and ERβ expression in salt-sensitive rats. Rats were subjected to sham surgery (intact), ovariectomy, and ovariectomy with estrogen replacement. Kidneys were harvested 8 weeks later. Western blot was used to measure ERα and ERβ expression in the cortex and medulla. In intact rats, ERα was 2.7- and 4.3-fold higher in salt-sensitive compared with salt-resistant rats in the renal cortex and medulla, respectively. In salt-sensitive rats, ovariectomy caused 42% and 52% decreases in ERα and 107% and 314% increases in ERβ in renal cortex and medulla, respectively. In salt-resistant rats, ovariectomy caused 33% and 150% increases in ERα and 107% and 100% increases in ERβ in renal cortex and medulla, respectively. Estrogen replacement did not alter ERα but restored ERβ expression levels similar to levels in intact rats in both salt-sensitive and salt-resistant rats. Thus, estrogen loss had opposite effects on ERα in salt-sensitive (downregulation) and salt-resistant rats (upregulation). We propose that the decrease in ERα expression in salt-sensitive rats after estrogen loss alters the balance of renal ERs and may play a role in accelerating the development of hypertension and renal damage. (Hypertension. 2007;50:768-772.)

Key Words: Dahl rats ■ salt sensitivity ■ hypertension ■ estrogens ■ estrogen receptors ■ kidney

The risk of cardiovascular disease has been found to be significantly increased in premenopausal women after surgically induced premature menopause.1 Hormone therapy seems to provide cardiovascular protection in this population of women.2 Similarly, our previous studies in female Dahl salt-sensitive (S) rats have confirmed that surgically induced menopause and estrogen depletion happen immediately on removal of the ovaries. We have observed that ovariec-to-mized S rats become hypertensive more rapidly than intact S rats and that 17-estradiol (E2) treatment delayed the development of hypertension.3,4

The mechanisms of this increased risk in hypertension in premenopausal women and its prevention by E2 replacement have not been fully elucidated, but it is feasible that the abrupt reduction in E2 levels can trigger complex changes in different organ systems resulting in functional and structural disturbances. We have demonstrated significant effects of E2 in preserving the morphology of the kidney during age-related increases in blood pressure.5 Central to these mechanisms are the estrogen receptors (ERs), to which estrogens bind. The ERs exists in 2 main forms, ERα and ERβ. They are products of different genes and exhibit tissue- and cell type–specific expression. Vascular cells express both; thus, this coexpression suggests different physiological roles for these receptors. ERs are expressed in varying numbers in both endothelium and vascular smooth muscle cells of multiple organ systems.6 A significant association between the number of ERs and normal endothelial cell function has been reported and suggests that a decreased number of endothelial ERs may represent a risk factor for cardiovascular disease.7 It has also been suggested that estrogen signaling is a dynamic balance between ERα and ERβ.8 ERα activation has been found to mediate vascular relaxation and may play a role in the protective effects of estrogens,9,10 whereas ERβ has been shown to be important in the inflammatory response to vascular injury.11,12 All of these data support an important role for both ERs in the vascular effects of estrogens and in the regulation of the vascular function.

In addition to estrogen loss, another risk factor that contributes to developing hypertension is salt sensitivity, which has been reported to increase after surgical menopause,13 although it has been demonstrated that women who develop postmenopausal hypertension are already salt sensitive before the onset menopause.14 Consistent with these findings in humans, we have shown that young S females become hypertensive after ovariectomy and that estrogen loss promotes glomerulosclerosis and tubular fibrosis in this model.3-5 Interestingly, the hypertension and renal damage...
associated with estrogen loss in S rats are observed while the animals are fed a low-salt diet. Thus studies in women and in S rats indicate that the genetic predisposition to salt-sensitive hypertension rather than a high-salt diet is crucial for the development of hypertension in response to estrogen loss.\(^3,4,13,14\) As a result, we have used the female S rat fed a low-salt diet to study the contribution of the kidney to the mechanisms of postmenopausal hypertension.

In the present study, we tested the hypothesis that estrogen depletion would change the expression of ER\(\alpha\) and ER\(\beta\) in the kidney of salt-sensitive rats. Our specific objective was to determine how E2 depletion and E2 replacement affect the ER\(\alpha\) and ER\(\beta\) expression in the kidney by subjecting young female S and Dahl salt-resistant (R) rats to ovarioectomy or E2 replacement treatment. Eight weeks after estrogen depletion or replacement, we evaluated ER expression levels using Western blot analysis in the renal cortex and medulla in young 4-month–old rats fed a low-salt diet.

**Methods**

**Experimental Protocol**

S and R (Rapp strain) female rats were purchased from Harlan Sprague-Dawley (Indianapolis, Ind) at 6 to 7 weeks of age, and they were maintained on a phytoestrogen-free, sodium-deficient diet (0.1% NaCl, Teklad 01409) for the duration of the study. One week after arrival, rats were anesthetized with isoflurane, and the ovaries were exposed (sham surgery) or removed (ovarioectomy) through bilateral flank incisions. The rats were divided into 3 groups: sham surgery intact, ovarioectomy (Ovx), and ovarioectomy with implantation of a silastic pellet containing E2 (Ovx+E). In those rats receiving estrogen replacement treatment, E2-filled (17\(\beta\)-estradiol, 5 mg, Sigma) silastic tubes 1 cm in length were implanted subcutaneously at the time of Ovx surgery. This study included a total of 6 groups: S intact (SIntact), S ovx (SOvx), S Ovx + E (SOvx+E), R intact (RIntact), R Ovx (ROvx), and R Ovx + E (ROvx+E). Each group had 6 rats. All of the rats remained in their home cage until they reached 4 months of age (8 weeks after Ovx surgery). At this age, a blood sample was obtained for estradiol measurement, and the kidneys and uterus were harvested and weighted. Immediately, the renal cortex and medulla were dissected and stored at –80°C until analyzed. The protocol was approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee and was performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

**Western Analysis**

The different tissue regions were homogenized on ice with a glass tissue homogenizer in 1 mL of lysis buffer containing 20 mmol/L Tris (pH 7.4), 2.5 mmol/L EDTA, 1% Triton, 10% glycerol, 0.1% sodium dodecyl sulfate, 10 mmol/L Na\(_2\)PO\(_4\), 50 mmol/L NaF, 1 mmol/L Na\(_3\)VO\(_4\), 1 mmol/L PMSF, 10 \(\mu\)g/mL leupeptin, and 10 \(\mu\)g/mL of aprotinin. Homogenates were centrifuged at 12 000 g for 20 minutes. The supernatant was diluted in sodium dodecyl sulfate sample buffer (0.5 mol/L Tris-HCl [pH 6.8], 10% vol/vol of glycerol, 10% wt/vol of sodium dodecyl sulfate, 5% vol/vol of \(\beta\)-mercaptoethanol, and 0.05% wt/vol of bromophenol blue). The protein concentration was determined colorimetrically with BSA as a standard, (Bio-Rad protein assay). One-hundred-microgram protein samples were size separated by SDS-PAGE (12%) and electroblotted to 0.45-\(\mu\)m/L polyvinylidene fluoride membranes. The membranes were incubated with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 for 1 hour, after overnight incubation with a 1:1000 dilution of primary antisera (ER\(\alpha\) or ER\(\beta\), Upstate), at 4°C overnight. The membranes were washed and incubated with 1:2000 dilution of goat anti-rabbit IgG and horseradish peroxidase-conjugated (Upstate), and then proteins were visualized by enhanced chemiluminescence. The densities of specific bands were quantified by densitometry using Scion software. Three sets of densitometric values by sample were used for the calculation. To verify equal loading of sample protein, the membranes used for ER\(\alpha\) and ER\(\beta\) were sequentially probed with a \(\beta\)-actin–specific antibody without stripping the membrane as described by Liao et al\(^6\). The Western blots were conducted in the linear range of the assay. We used ovary homogenate as a positive control for ER\(\alpha\) and F9 cell lysate (Santa Cruz, sc2245) as a positive control for ER\(\beta\).

**Plasma Estradiol**

Plasma estradiol levels were analyzed by radioimmunoassay (Ultra-Sensitive Estradiol kit, Diagnostic Systems Laboratories).

**Statistical Analysis**

Data are shown as mean±SEM. Overall differences among the groups were evaluated by 2-way ANOVA for 2 factors (salt sensitivity and estrogen status). Individual comparisons were evaluated by 1-way ANOVA followed by Tukey’s multiple comparison test. Statistical significance was assumed at the P<0.05 level.

**Results**

The ER\(\alpha\) expression levels in renal cortex and medulla are shown in Figure 1A and 1B, respectively. ER\(\alpha\) expression level was significantly higher in SIntact compared with RIntact rats in both the cortex (2.7-fold increase) and medulla (4.3-fold increase). In the S group, Ovx significantly decreased cortical and medullary ER\(\alpha\) expression levels by 42% and 52%, respectively (P<0.05 versus SIntact). In contrast, Ovx in the R group increased the cortical and medullary ER\(\alpha\) expression level by 33% and 150%, which was significant only in the medulla (P<0.05 versus RIntact). Estrogen replacement in Ovx animals had no effect on the cortical and medullary ER\(\alpha\) expression level in the S and R groups, because there were no differences between the Ovx and Ovx+E groups.

The ER\(\beta\) expression levels in the renal cortex and medulla are shown in Figure 2A and 2B, respectively. ER\(\beta\) expression level was not different between the SIntact group and RIntact group in both cortex and medulla. In the S group, Ovx significantly increased cortical and medullary ER\(\beta\) expression levels by 107% and 314%, respectively, and in the R group by 107% and 100%, respectively. Estrogen replacement in Ovx animals prevented the cortical ER\(\beta\) upregulation in both S and R rats. However, in the medulla, estrogen replacement prevented ER\(\beta\) upregulation only in S kidneys and not in R kidneys.

The effects of Ovx and Ovx+E on body weight (BW), kidney weight (KW), uterine weight (UW), and plasma estradiol levels are shown in the Table. BW measurements show that S rats are heavier than R rats. Ovx in the S and R rats resulted in an increase in the BW (24.5% and 22%, respectively) that was statistically significant (P<0.05). In contrast, E2 replacement in both S and R rats resulted in a decrease in BW that was statistically significant (P<0.05). KW measurements indicate that S rats had larger kidneys than R rats. In both S and R rats, KW was not affected by Ovx or Ovx+E. The UW and plasma estradiol levels were statistically decreased by Ovx in both S and R groups (P<0.001), whereas estrogen replacement prevented the decrease in UW and plasma estradiol.
Discussion

In this study, we evaluated how chronic E2 depletion and E2 replacement affect the ERα/H9251 and ERβ/H9252 expression levels in the renal cortex and medulla of young S and R rats fed a low-salt diet. The main findings are summarized as follows. First, the ERα expression level in kidney was higher in SIntact rats than in RIntact rats. Second, in S rats, Ovx caused a decrease in renal ERα expression and an increase in renal ERβ expression, but in R rats, Ovx caused an increase in renal ERα expression and an increase in renal ERβ expression. Third, estrogen replacement in Ovx animals did not alter ERα expression but restored ERβ expression levels similar to intact rats in both S and R rats. These findings demonstrated that estrogen loss had opposite effects on renal ERα expression in low-salt–fed salt-sensitive (down-regulation) and salt-resistant rats (upregulation) and suggest that ERα is important in protecting the salt-sensitive kidney against renal damage even under low-salt conditions.

We have reported previously that at 4 months of age SIntact rats have similar blood pressures as SOvx+E rats (122 ± 3 and 121 ± 2 mm Hg, respectively), but blood pressure in SOvx rats is significantly higher (131 ± 3 mm Hg). Therefore, at 4 months of age, estrogen loss in low-salt–fed S rats has affected blood pressure but has not yet affected renal function and morphology. Measurements of plasma estradiol and UW in the present study confirm the effectiveness of Ovx and estrogen treatment in depleting and restoring circulating estrogen levels. Ovx caused increases in BWs in both S and R rats, but KWs were not affected. Similarly, estrogen replacement caused decreases in BWs in both groups without affecting KWs.

The results of the present study reveal that at 4 months of age, low-salt–fed SIntact rats have higher levels of ERα but
similar levels of ERβ compared with RIntact rats. The cause of the upregulation in baseline ERα in Intact S rats is not known, but we speculate that the upregulation in ERα protein in S rats is a mechanism for renal protection. In other words, the genetic predisposition to salt sensitivity is associated with an upregulation in ERα to help maintain normal renal function in the genetically altered salt-sensitive kidney.

Ovx had opposite effects on ERα protein expression in S and R rats. In the S rats, Ovx resulted in a decrease in ERα protein, whereas in the R rats, Ovx resulted in an increase in ERα protein. In both S and R rats, estrogen replacement did not reverse the effects of Ovx on ERα expression. This is an interesting finding, because this implies that renal ERα protein expression depends on female sex hormones but is independent of circulating estrogen levels. Because progesterone was not replaced in estrogen-treated animals, it is possible that the effects of Ovx on ERα were mediated via progesterone. The ability of progesterone to regulate ER expression has been demonstrated in uterine tissue. The effects of Ovx on ERβ protein expression were similar in S and R rats. Ovx caused an increase in ERβ expression, and estrogen replacement reversed this effect in both groups. Thus, the alterations in ERβ were dependant on circulating estrogen levels and independent of the genetic predisposition to salt sensitivity. ERβ has been abundantly localized in vascular smooth muscle cells and has also been reported to have an important role in response to vascular injury in premenopausal and postmenopausal women. In addition, ERβ expression has been correlated with the degree of atherosclerosis. Therefore, we suspect that upregulation in ERβ expression observed in our study was in response to renal injury induced by Ovx.

ERα and ERβ expression in the renal cortex has been reported in female Sprague Dawley rats by Rogers et al. They observed that Ovx was associated with a decrease in ERα protein expression, which was reversed with estrogen replacement. No effect of Ovx was observed on ERβ expression. The downregulation of ERα by Ovx is consistent with our observations in S rats. However, we did not observe the reversal of the effect with estrogen treatment. The lack of effect of estrogen treatment on ERα in the present study was not because of low plasma levels of estrogen, because the levels in Ovx+E were higher than intact rats. The reasons for the different effects of estrogen treatment are not clear but may be a result of genetic differences in the animal strains (Sprague-Dawley versus S).

We propose that the decrease in ERα expression in salt-sensitive rats after ovariectomy contributes to the acceleration in the development of hypertension and renal damage observed previously in these animals. Our data suggest that Ovx in R rats elicits a normal upregulation of renal ERα protein in response to estrogen loss. In contrast, Ovx in S rats elicits an abnormal downregulation of renal ERα protein, which is essential in maintaining renal function and protecting against renal damage. Our previous evaluation of glomerulosclerosis and tubulointerstitial fibrosis in similar groups of Ovx rats (4-month–old intact, Ovx, and Ovx+E) revealed that, at this age, renal damage was not observed in Ovx rats; however, the detrimental effects of Ovx on renal morphology were clearly evident in older 12-month–old rats. Interestingly, other models of renal insufficiency, as observed with diabetes, are associated with a decrease in circulating estrogen levels and a decrease in renal ER protein expression.

A mechanism by which estrogen may be protective in renal injury is by reducing oxidative stress via NO production. ERα has shown to be involved in the production of NO, and a decreased production of NO has been implicated in the pathogenesis of salt sensitivity. The area of the kidney may also be important in determining the effect of estrogen loss in the kidney. The renal medulla has more NO synthase activity than renal cortex, which implies a greater potential to produce NO than the cortex. The renal medulla is more susceptible to the changes in the reduction-oxidative balance. It is well known that the kidney is sensitive to oxidative stress. Studies indicate that exaggerated oxidative stress in renal medulla might be an important pathogenic mechanism resulting in hypertension. It has also been reported that oxidative stress induced in macrophages affects the expression of ERα and ERβ differently.

**Perspectives**

The mechanism of postmenopausal hypertension is not well understood. It has been proposed that salt sensitivity is a contributing factor. By using the S rat as a model of postmenopausal hypertension, we have gained insights into how salt sensitivity and estrogen loss (as observed with menopause) interact to set the stage for hypertension. The current study revealed that estrogen loss in salt-sensitive animals (but not salt-resistant animals) will decrease ERα expression in the kidney. We propose that women who are genetically predisposed to salt-sensitive hypertension are protected against hypertension and renal damage before menopause via an overexpression of renal ERα protein. After menopause, the loss of circulating estrogen and the resultant downregulation of ERα expression accelerate the development of hypertension and renal damage. Future studies will evaluate the effect of age on ERα and ERβ in S rats. In addition, we will investigate how salt sensitivity, age, and ER imbalance affect oxidative stress and inflammation at the

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**Table. BW, KW, Plasma E2, and UW of S and R Rats in Intact, Ovx, and Ovx+E Groups**

<table>
<thead>
<tr>
<th>Variable</th>
<th>SIntact</th>
<th>SOvx</th>
<th>SOvx+E</th>
<th>RIntact</th>
<th>ROvx</th>
<th>ROvx+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>281.5±4.1</td>
<td>350.5±11.3*</td>
<td>241.6±5.0*</td>
<td>253.5±4.9</td>
<td>309.7±13.4†</td>
<td>209.5±5.3†</td>
</tr>
<tr>
<td>KW, g</td>
<td>1.77±0.04</td>
<td>1.80±0.03</td>
<td>1.65±0.09</td>
<td>1.57±0.04</td>
<td>1.53±0.08</td>
<td>1.53±0.05</td>
</tr>
<tr>
<td>E2, pg/mL</td>
<td>15.7±1.7</td>
<td>7.3±0.5*</td>
<td>21.2±3.2</td>
<td>17.1±4.6</td>
<td>7.6±0.7†</td>
<td>42.9±12.6†</td>
</tr>
<tr>
<td>UW, g</td>
<td>0.44±0.02</td>
<td>0.09±0.01*</td>
<td>0.44±0.01</td>
<td>0.41±0.02</td>
<td>0.09±0.01†</td>
<td>0.46±0.01</td>
</tr>
</tbody>
</table>

*P<0.05 vs SIntact; †P<0.05 vs RIntact.
levels of the renal cortex and renal medulla to promote hypertension. The ultimate goal of these studies is to understand the mechanisms of postmenopausal hypertension and to identify the associated risk factors in an effort to develop more effective treatments for this disease.

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Disclosures

None.

References

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