Chronic Estrogen Treatment in Female Transgenic (mRen2)27 Hypertensive Rats Augments Endothelium-Derived Nitric Oxide Release

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Postmenopausal estrogen replacement therapy is associated with a reduction in cardiovascular events in women, but the mechanisms for this protection are unclear, especially in hypertensive subjects. In this study we investigated the effects of 17β-estradiol (E2) treatment on blood pressure and endothelial function of transgenic [(mRen2)27] hypertensive and normotensive rats. Thirty female transgenic negative [Tg(−)] and hypertensive positive [Tg(+)] rats were ovariectomized and received either E2 (1.5 mg/rat, subcutaneously, for 3 weeks) or placebo. Chronic 17β-estradiol treatment lowered mean blood pressure in both Tg hypertensive (159 ± 4 vs 145 ± 4 mm Hg, \(P < 0.05\), placebo vs E2) and normotensive rats (119 ± 4 vs 108 ± 2 mm Hg, \(P < 0.05\), placebo vs E2). Pressor responses to intravenous injection of phenylephrine were augmented in the Tg(+) as compared with Tg(−) rats. With chronic E2 treatment the pressor responses to phenylephrine were attenuated in both groups. Isometric tension of aortic rings was measured in vitro in organ chambers. The acetylcholine (Ach)-induced endothelium-dependent vascular relaxation was less potent in Tg(+) versus Tg(−) rats. E2 treatment significantly enhanced the Ach-induced relaxation of both Tg(+) and Tg(−) groups (ED50: 55.5 ± 11.7 vs 10.3 ± 2.6; 23.8 ± 6.5 vs 5.1 ± 1.2 nmol/L, placebo vs E2 in Tg(+) and Tg(−), respectively).

After E2 treatment the ED50 response in Tg(+) rats was no different from Tg(−) rats. However, the maximum vasodilation elicited by Ach was attenuated in Tg(+) as compared with Tg(−) rats. The calcium ionophore (A23187)-induced endothelium-dependent relaxation was less potent in Tg(+) as compared to Tg(−) rats and was enhanced by E2 treatment only in Tg(+) animals. There were no differences in the vasodilator responses elicited by sodium nitroprusside. Removal of endothelium and blockade of NO production abolished the endothelium-dependent vasodilation. The selective NO synthase inhibitor, \(N^G\)-monomethyl-L-arginine (LMMNA), was used to evaluate indirectly the basal contribution of NO in vascular rings. The response to LMMNA was attenuated in untreated Tg(+) as compared to Tg(−) rats. E2 treatment augmented the contraction response to NOS inhibition in both Tg(+) and Tg(−) rats, resulting in a response in Tg(+) rats that was no different from Tg(−) rats. These results indicate that untreated, surgically ovariectomized hypertensive rats show deficiencies in endothelial function, which can be improved by estrogen replacement. Am J Hypertens 1997;10:662–670 © 1997 American Journal of Hypertension, Ltd.

KEY WORDS: Estrogen, endothelium, renin gene, transgenic rats, post-menopausal, hormone replacement, vascular reactivity, nitric oxide.
Epidemiological studies indicate a relatively lower incidence of cardiovascular events in premenopausal women as compared with age-matched men. This difference lessens with the onset of menopause and, within a decade, the number of cardiovascular events in postmenopausal women is as high or higher than in men. While it is accepted that estrogen replacement therapy is associated with a 50% reduction in the risk of cardiovascular disease in postmenopausal women, the mechanisms affording this cardiac and vascular protection remain poorly understood. Differences in lipoprotein metabolism, insulin levels, fibrinolytic activity, and blood pressure have been advanced as possible explanations for estrogen-induced cardioprotection. However, these mechanisms do not provide a sufficient explanation for the beneficial effects of estrogen on the cardiovascular system.

Recent studies have suggested that estrogen-induced cardioprotection may be mediated by an increase in the production of vascular nitric oxide (NO). Hayashi et al demonstrated that the basal release of NO from aortic rings of female rabbits is greater than in males and correlates with circulating estradiol levels. Estradiol increases the expression of constitutive NO synthase in tissues in vivo and cultured endothelial cells in vitro. Estrogen treatment improves endothelium-dependent vasodilation of coronary and peripheral arteries to acetylcholine while it also increases circulating NO (nitrite/nitrate) levels in postmenopausal women and surgically ovariectomized monkeys.

In animal models of hypertension chronic estrogen treatment partially reverses the diminished endothelium-dependent relaxation and attenuates the magnitude of the blood pressure rise in spontaneously hypertensive rats (SHR). In keeping with these findings we have demonstrated an amplification of nitric oxide dependent vasodilation in transgenic hypertensive rats harboring the mouse Ren-2 gene. Moreover, a pronounced sexual dimorphism of blood pressure can be demonstrated between age-matched male and female transgenic rats. With this in mind, we have evaluated the effects of chronic 17β-estradiol treatment on blood pressure and vascular endothelial function of age-matched female transgenic positive [Tg(+)] hypertensive rats as compared to their normotensive transgenic negative [Tg(−)] controls.

### MATERIALS AND METHODS

#### Surgical Procedures
Following approval by the Institutional Animal Care and Use Committee, thirty 12 week old, heterozygous female Tg(−) and hypertensive Tg(+) rats (body weight: 220 to 250 g) from the Hypertension Center Transgenic Animal Research Colony of the Bowman Gray School of Medicine were anesthetized with ketamine (30 mg/kg, intramuscularly) and xylazine (5 mg/kg, intramuscularly) and bilaterally ovariectomized. The animals were implanted subcutaneously with pellets containing either 17β-estradiol (E2) (1.5 mg/rat, for 3 week release) or placebo (Innovative Research of America, Toledo, OH); animals were randomized into four groups: ovariectomized Tg(+) / placebo, ovariectomized Tg(+) / E2, ovariectomized Tg(−) / placebo, and ovariectomized Tg(−) / E2. After recovery, rats were allowed free access to water and fed normal powder chow providing 17 mEq of NaCl and 28 mEq of K+ per 100 g of solid weight (Rodent Laboratory Chow 5001, Purina Mills Inc., Richmond, IN). The animals were housed individually in plastic cages in a room maintained at 22°C and lighted for 12 h.

#### Experimental Protocols
At the end of the 3 week period of hormone replacement, rats were again anesthetized with ketamine and xylazine, and a polyethylene catheter (PE-50, Clay Adams, Becton-Dickinson, Franklin Lakes, NJ) was implanted into the abdominal aorta via the right femoral artery. Another plastic catheter was placed into the inferior vena cava through the right femoral vein. The free end of both catheters was tunneled to the back of neck as described previously. All procedures were performed under sterile condition and followed by an injection of a prophylactic dose of antibiotics (Penicillin G, 30,000 U intramuscularly). The animals were allowed 2 days of recovery. The arterial catheter was connected to a solid strain-gauge microtransducer (MP-150, Micron Instruments Inc., Los Angeles, CA). Blood pressure was determined by a PC-based data acquisition program developed in our laboratory, and the arterial pressure waveform was also displayed in a polygraph (Model 7, Grass Instruments Inc., Quincy, MA). After a 1-h stabilization period, dose-dependent phasic pressor responses to intravenous injections of phenylephrine (doses: 2, 5, 10 μg) were obtained in conscious, freely moving animals.

On the day following blood pressure measurements, vascular reactivity was evaluated in isolated vessels mounted in organ chambers. Rats were killed by decapitation, and blood was collected for the determination of plasma estradiol levels using a commercially available radioimmunoassay kit (SeraCare Diagnostics Inc., Allentown, PA).

For vascular reactivity studies, the thoracic aorta was...
removed and dissected free of connective tissues. The vessels were cut into 3 mm rings and mounted in organ chambers filled with modified Krebs-Henseleit buffer (composition in mmol/L: NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, CaNaEDTA 0.026, and glucose 11). The solution was aerated with 95% O₂ and 5% CO₂ at 37°C (pH 7.4) and contained 10 μmol/L indomethacin to prevent the production of vasoactive prostaglandins throughout the experiment. In some rings, the endothelium was denuded by gentle mechanical rubbing with a stainless steel wire. The isometric tension was measured continuously by using a polygraph (Model 7, Grass Instrument Inc., Quincy, MA). Basic tension was set at 2 g after a 60 min equilibration period. In preliminary experiments, we established that 2 g of tension was optimal for both normotensive and hypertensive rat aorta rings after repeated exposure to 40 mmol/L KCl. Endothelial integrity was tested using 10⁻⁶ mol/L acetylcholine (Ach) in rings preconstricted with 10⁻⁶ mol/L phenylephrine (PE). The presence of a functional endothelium was associated with more than 60% relaxation to Ach, whereas the absence of relaxation using the same dose of Ach indicated effective removal of the endothelium. The endothelium-dependent relaxation curves of Ach (10⁻¹⁰ to 10⁻⁴ mol/L), and the calcium ionophore, A23187 (10⁻¹⁰ to 10⁻⁶ mol/L) were obtained in intact rings preconstricted with 10⁻⁷ mol/L PE. The contribution of the endothelium to the dosee-dependent vasodilation was investigated in the rings with denuded endothelium or pretreated with NO synthase inhibitor l-nitro-arginine methyl ester (L-NAME, 10⁻⁴ mol/L) for 30 min. To determine whether estrogen replacement modulates the vasoactivity of vascular smooth muscle cells in both normotensive and hypertensive rat aorta, relaxation–response curves to sodium nitroprusside (SNP, 10⁻¹⁰ to 10⁻⁷ mol/L) were also evaluated in aortic rings preconstricted with PE (10⁻⁷ mol/L). An indirect estimate of basal release of NO from intact vascular rings was evaluated, as previously described by Frew et al.²⁶ Vessels were mildly constricted with PE (3 × 10⁻⁹ mol/L) and then exposed to the NO synthase inhibitor N⁶-monomethyl-L-arginine (L-NMMA, 10⁻⁴ mol/L), which was demonstrated previously to be selective for blocking basal NO release in the rat aorta.²⁶ Denuded rings and those pretreated with L-NAME (10⁻⁴ mol/L, 20 min) were preconstricted with PE (10⁻⁸ mol/L) to achieve a similar degree of vascular tone, because removal of endothelium and blockade of NO synthase with L-NAME enhance the constriction induced by PE. A 60 min equilibration period was allowed between responses.

**Chemicals** N⁶-Monomethyl-L-arginine was purchased from Calbiochem (La Jolla, CA), and all other drugs were obtained from Sigma Chemical Co. (St. Louis, MO). For intravenous injection, phenylephrine was prepared in 0.9% saline daily. Other solutions were prepared in distilled water and stored at −20°C in stock solution. A23187 was dissolved in dimethyl sulfoxide and indomethacin was dissolved in 0.2 mol/L Na₂CO₃ stock and diluted in Krebs buffer. Drug concentrations were expressed as final molar (mol/L) concentration in organ chamber.

**Statistical Analysis** Vascular relaxation was expressed as percentage (%) of isometric tension of rings preconstricted with 10⁻⁷ mol/L PE and constriction was normalized as percentage (%) of 80 mmol/L KCl-induced maximal contraction. The concentration of drugs inducing 50% (ED₅₀) of the maximal relaxation was calculated using a nonlinear regression method (Sigmoid curve fitting program, PRISM, San Diego, CA). All values are mean ± SEM (standard error of mean). One way analysis of variance (ANOVA) followed by Neuman-Keul’s test for multiple comparisons was used for statistical comparison. In addition, the Student’s t test for unpaired observation for blood pressure, heart rate, and 17β-estradiol levels was used for statistical analysis. A P value of < .05 was considered statistically significant.

**RESULTS**

The baseline levels of mean blood pressure and heart rate of conscious freely moving Tg (+) and Tg (−) rats at the end of 3 weeks of estrogen replacement therapy or placebo are summarized in Table 1. Mean blood pressure of ovariectomized female Tg (+) rats was higher than that recorded in normotensive controls regardless of whether animals were exposed to estrogen replacement. Chronic 17β-estradiol treatment lowered mean blood pressure in normotensive rats and attenuated the magnitude of the blood pressure rise in Tg (+) rats. Heart rate was not changed with estrogen replacement therapy. Plasma 17β-estradiol levels were 214 ± 33 pg/mL and 166 ± 20 pg/mL for E₂ treated Tg (+) and Tg (−) groups, respectively, and less than 15 pg/mL for both placebo groups.

**Estrogen Attenuates Phenylephrine Mediated Vascular Constriction** Chronic 17β-estradiol treatment significantly attenuated the pressor responses to intravenous injection of PE in Tg (+) hypertensive (P < .01) and Tg (−) normotensive rats (P < .05) (Figure 1A and 1B) in a dose-dependent manner, except at the highest

**TABLE 1. EFFECTS OF CHRONIC ESTROGEN TREATMENT ON BASAL MEAN BLOOD PRESSURE AND HEART RATE**

<table>
<thead>
<tr>
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<th>Tg(−)/P</th>
<th>Tg(−)/E₂</th>
<th>Tg(+)/P</th>
<th>Tg(+)/E₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mm Hg)</td>
<td>119 ± 4</td>
<td>108 ± 2*</td>
<td>159 ± 4†</td>
<td>145 ± 4†</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>382 ± 12</td>
<td>342 ± 9</td>
<td>367 ± 9</td>
<td>385 ± 21</td>
</tr>
</tbody>
</table>

* P < .05, compared with placebo treated; † P < .01, compared with similarly treated Tg(−) rats.
dose of PE in normotensive rats. Placebo treated Tg(+) rats showed markedly higher pressor responses to intravenous injections of PE as compared with similarly treated Tg(-) rats ($P < .01$). The hyperresponses to PE in Tg(+) rats were abolished after estrogen treatment, as there was no significant difference in the PE responses between estrogen treated Tg(+) and Tg(-) animals.

**Estrogen Treatment Modulates Endothelium Dependent and Independent Vasodilation** The Ach-induced endothelium-dependent vascular relaxation in thoracic aortic rings preconstricted with $10^{-7}$ mol/L PE was less potent in Tg(+) as compared with normotensive rats ($ED_{50}$ 23.8 ± 6.5 vs 55.5 ± 11.7 nmol/L, $P < .05$, Tg(-) v Tg(+)). Estrogen replacement significantly enhanced Ach-induced relaxation of both hypertensive and normotensive rats (Figure 2). There was a leftward shift of the $ED_{50}$ 5.5-fold in Tg(+) and 4.5-fold in Tg(-) rats (Table 2). After E2 treatment, there was no significant difference in the $ED_{50}$ values of Tg(+) and Tg(-) rats. In addition, E2 replacement enhanced the maximum vasodilation elicited by Ach in both Tg(+) and Tg(-) rats. The maximum responses to Ach were significantly less in Tg(+) hypertensive rats as compared to similarly treated normotensive controls (Table 2).

The non-receptor-mediated, endothelium-dependent vasodilation induced by the calcium ionophore, A23187 was less potent in Tg(+) as compared with Tg(-) rats. E2 treatment increased the A23187-induced vasodilation only in Tg(+) rats. The $ED_{50}$ of Tg(+) / E2 was shifted leftward by sixfold, whereas there was no difference in the $ED_{50}$ of Tg(-) animals treated with either placebo or E2 (Figure 3). Removal of the endothelium and pretreatment with the NO synthase inhibitor L-NAME ($10^{-4}$ mol/L) abolished the endothelium-dependent vasodilation of all four groups of animals (six to eight rings from five to seven rats, data not shown). There were no differences in the vasodilator responses elicited by the NO donor, sodium nitroprusside, between Tg(+) hypertensive and normotensive animals regardless of the presence of estrogen (Figure 4).

**Effects of Estrogen Treatment on Basal NO Activity in Vascular Rings** As an indirect measurement of basal NO release, mildly preconstricted aortic rings were exposed to the NO synthase inhibitor L-NMMA. In the absence of estrogen, the increase in contraction following L-NMMA was significantly less in Tg(+) as compared with normotensive, placebo-treated animals ($F = 10$, $P < .05$, ANOVA, Tg(+) / placebo v Tg(-) / placebo) (Figure 5). Chronic estrogen treatment resulted in a significant enhancement of the contractions of aortic rings exposed to L-NMMA, suggesting that E2 treatment increases the basal release of NO from both hypertensive and normotensive animals. There was no difference in the maximal contraction reached between the Tg(+) and Tg(-) estrogen-treated rats. Either removal of the endothelium or pretreatment with L-NMA ($10^{-4}$ mol/L) abolished the responses (six to eight rings from five to seven rats in each group; data not shown).

**DISCUSSION**

The present study demonstrates that chronic estrogen replacement was effective in reducing blood pressure in both normotensive and hypertensive female rats. Without estrogen replacement therapy, hypertensive rats demonstrate a reduced basal capacity to release
FIGURE 2. Dose-response curve of Ach on relaxation of rat aortic rings. E$_2$ treatment enhanced the acetylcholine-induced endothelium-dependent vascular relaxation causing a leftward shift of the ED$_{50}$ by 5.5-fold in Tg(+) and by 4.5-fold in Tg(-) rats. In addition, E$_2$ treatment enhanced the maximum vasodilation elicited by Ach in both Tg(+) and Tg(-) rats. Each group included 12 to 14 rings from six to eight rats. Values are mean ± SEM.

TABLE 2. EFFECTS OF CHRONIC 17β-ESTRADIOL TREATMENT ON THE Ach-INDUCED ENDOTHELIAL-DEPENDENT RELAXATION IN RAT AORTA

<table>
<thead>
<tr>
<th></th>
<th>Tg(-)/P</th>
<th>Tg(-)/E$_2$</th>
<th>Tg(+)/P</th>
<th>Tg(+)/E$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED$_{50}$ (nmol/L)</td>
<td>23.8 ± 6.5</td>
<td>5.1 ± 1.2†</td>
<td>55.5 ± 11.7‡</td>
<td>10.3 ± 2.6†</td>
</tr>
<tr>
<td>Max. relaxation (%)</td>
<td>90 ± 2</td>
<td>98 ± 1*</td>
<td>71 ± 5‡</td>
<td>82 ± 4‡</td>
</tr>
</tbody>
</table>

* P < .05, and † P < .05 compared with placebo treated rats.  
‡ P < .01, compared with similarly treated Tg(-) rats.
pression of the renin gene activates a buffering response from the vascular endothelium that is significantly greater in Tg hypertensive as compared to normotensive animals. In the present studies, however, a diminished endothelium contribution of both basal and stimulated nitric oxide was found in aortic rings from Tg(+) rats, as measured by L-NMMA-induced constriction and Ach-induced relaxation of aortic rings. Differences in the whole animal and the in vitro responses after nitric oxide blockade could be the resultant of NO’s effects in different vascular beds, the compounding influence of flow and pressure on release of nitric oxide in the whole animal, and actions within the central and peripheral nervous system. A selective defect in the coronary endothelium of young transgenic rats was demonstrated by Tschudi et al, who showed that a greater basal capacity to release NO was accompanied with no change in acetylcholine-induced relaxation. In adult animals the basal capacity to release NO from the coronary arteries was significantly smaller in transgenic animals as compared to normotensive controls, a finding that is in agreement with the responses found in thoracic vessels taken from adult animals in our study. While different mechanisms may be activated by endogenous inhibition of NO, the current experiments and those previously published demonstrate a consistent effect of estrogen replacement therapy in potentiating the vasodilator actions of NO, both in vivo and in isolated blood vessels.

Nitric oxide, as a potent endothelium-derived vasodilator, has a strong impact on the regulation of blood
FIGURE 5. Treatment of preconstricted vessels with L-NMMA, the selective NOS inhibitor, provides an indirect measure of the contribution of basal NO release. E2 treatment caused greater and more prolonged contraction of preconstricted aortic rings to L-NMMA in both Tg(+) and Tg(−) groups, as compared with placebo treated rats. Each group included 12 to 14 rings from six to eight rats. Values are mean ± SEM.

pressure and vascular tone. Abnormalities in endothelial production of NO occur in atherosclerosis and hypertension. Huang et al. demonstrated that mice lacking the gene for endothelial NO synthase develop hypertension. In these animals, Ach-induced endothelium-dependent vasodilation was absent. In the present study, the effects of estrogen on endothelial production of NO were measured by assessing endothelium-dependent vasodilation by Ach in the thoracic aorta of normotensive and transgenic rats. The hypertensive animals when left untreated, however, showed a lesser degree of maximal dilatory capacity to Ach, a reduced potency to Ach, and less ability to release NO, when compared to untreated normotensive animals. The ED50 to Ach, the maximal dilatory response, and the basal release of NO were markedly enhanced by chronic treatment with estrogen in both normotensive and hypertensive animals. The fact that the response to L-NMMA was increased to a similar extent in Tg(+) rats as compared with Tg(−) rats in the presence of estrogen suggests a greater enhancement of basal production of NO in the Tg(+) rats, in light of their lower basal level of NO without estrogen. These findings are in agreement with previous studies, which showed that estrogen increases the expression of endothelial NO synthase. Our studies further illustrate that a number of abnormalities in the hypertensive rats can not be entirely corrected by estrogen. The maximal dilatory response to Ach in the Tg(+) rats, although enhanced by estrogen, was not entirely normalized when compared to estrogen-treated, normotensive rats. On the other hand, use of the calcium ionophore uncovered a deficiency in untreated, hypertensive rats that was corrected in the presence of estrogen to values no different than the responses in normotensive control.

The action of estrogen on the endothelium-dependent vasorelaxation is not likely to be mediated by endothelium-derived hyperpolarizing factor (EDHF), as NO synthase inhibition completely abolished the augmented response. In addition, because all vessels in this study were treated with indomethacin, a participation of prostaglandins was excluded. The contribution of non-receptor-mediated endothelium dependent relaxation, by the calcium ionophore, A23187, was found to be influenced by estrogen only in the Tg(+) hypertensive rats. The lack of effect on the non-receptor-mediated relaxation in normotensive animals is in agreement with previous studies by Gisclair et al. Estrogen may act at a post-receptor site, imparting additional improvement of endothelial function in transgenic hypertensive animals. Because the relaxation sensitivity to sodium nitroprusside was unchanged with either estrogen treatment or hypertension, our results would indicate that the response of the vascular smooth muscle cells to EDRF / NO is not influenced by estrogen or exposure to high blood pressure.

Another observation made in these studies was that estrogen enhances the basal release of NO from the endothelium of Tg(+) and Tg(−) rats. As an indirect measure of basal release of NO, the time-dependent contractions in vessels treated with the NOS inhibitor, L-NMMA, have been used as an estimate of basal release of NO in arteries from rats, rabbits, pigs, and coronary arteries of transgenic rats. In comparison to vessels from Tg(−) rats treated with placebo, hypertensive rats without estrogen have a reduced endothelium dependent contraction, indicative of a lower basal release rate of NO and suggesting that hypertension itself impairs the basal release of NO from thoracic aorta endothelium. Estrogen markedly augmented the constriction of aortic rings to NO synthase inhibitors in both Tg(+) and Tg(−) rats, reach-
ing similar levels. These findings indicate that, although estrogen can augment the basal release of NO in both normotensive and hypertensive animals, there appears to a greater enhancement of basal NO production in hypertensive rats.

In summary, estrogen replacement therapy reduces blood pressure in both hypertensive and normoten-sive animals. The endothelium from vessels of un-treated, surgically postmenopausal Tg(+) hyperten-sive rats shows greater abnormalities in receptor- and non-receptor-mediated vasodilatory events and in the basal capacity to release NO. Estrogen treatment improves endothelial function in both normotensive and hypertensive animals and corrects most endothelial-dependent deficiencies of the hypertensive rats.

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REFERENCES


