Estrogen or the AT1 Antagonist Olmesartan Reverses the Development of Profound Hypertension in the Congenic mRen2.Lewis Rat

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Abstract—The influence of estrogen on the regulation of cardiovascular function remains a controversial and complex area of investigation. We assessed the effects of estrogen depletion in the congenic mRen(2).Lewis rat, established from the back-cross of the original (mRen2)-27 transgenic onto the Lewis inbred strain. Ovariectomy of heterozygous mRen(2).Lewis at 4 to 5 weeks resulted in a progressive increase in blood pressure compared with the sham surgery congensics at weeks 6 to 11. At 11 weeks, the ovariectomized mRen(2).Lewis (OVX) systolic blood pressure averaged 195±3.7 mm Hg versus 141±0.0 mm Hg for sham. Plasma Angiotensin (Ang) II, serum ACE activity, plasma renin concentration, as well as urinary excretion of Ang II, 8-isoprostane F_2alpha, and endothelin-1 were elevated; however, renal mRNA levels of eNOS were suppressed after ovariectomy. Estrogen replacement reduced blood pressure below both the sham and OVX by 11 weeks (125±2.9 mm Hg, n=7, P<0.01 versus OVX and sham). Moreover, the AT1 receptor antagonist olmesartan (CS866; week 12 to 16) essentially normalized blood pressure to 113±5.4 mm Hg (n=6, P<0.01 versus OVX and sham). The attenuation of the hypertension was still evident 7 weeks after complete withdrawal of treatment (124±4.1 mm Hg at week 23). In summary, the OVX mRen.2.Lewis exhibited a rapid and sustained increase in blood pressure. Estrogen or olmesartan lowered pressure by a similar extent. We conclude that the ovary exerts considerable influence on the regulation of the blood pressure in the mRen2.Lewis strain, possibly by limiting activation of the renin-angiotensin system. (Hypertension. 2003;42[part 2]:781-786.)

Key Words: angiotensin II ■ angiotensin-converting enzyme ■ estrogen ■ rats, transgenic ■ hypertension, genetic

It is well recognized that ovarian hormones, particularly estrogen, exhibit a protective role on the cardiovascular system. After menopause, there is increased arterial pressure and higher rates of hypertension, renal disease, cardiovascular events, and mortality rates in women. However, recent clinical studies have questioned the beneficial effects of hormone replacement therapy (estrogen/progestin), particularly the actions that influence the cardiovascular system. Indeed, one arm of the Women’s Health Initiative (WHI) study with the combined therapy was halted prematurely because of negative outcomes, although the estrogen replacement group has continued at the present time. In light of the clinical data, the protective actions of estrogen would appear to be in contrast to the hormone’s effects on several components of the renin-angiotensin system (RAS)—one of the key regulatory systems for the control of blood pressure and cardiovascular pathologies. Specifically, estrogen stimulates the expression of angiotensinogen from the liver, kidney, and other tissues, as well as increases renin activity. However, estrogen has also been shown to suppress components of the RAS, including ACE and the AT1 receptor, perhaps achieving an overall balance to attenuate the development of hypertension and progression of other cardiovascular events.Various hypertensive models have marked gender differences in the expression of hypertension. Although the majority of the studies have focused on the relation of androgens to the development of blood pressure, there clearly remains strong evidence for the contribution of estrogens or their metabolites in cardiovascular regulation. For example, homozygous and heterozygous (mRen2)-27 male rats have blood pressures ~100 and 50 mm Hg higher, respectively, than their female littermates. We and other groups extensively use the (mRen2)-27 transgenics to study the mechanisms of renin overexpression and the development of hypertension. However, to eliminate the variability of the outbred SD background, (mRen2)-27 transgenics were back-crossed into the inbred Lewis rat, creating the new mRen(2).Lewis strain. After 9 generations, characterization of the mRen(2).Lewis rat revealed essentially the same degree of hypertension as the original transgenics, but the congenics lacked the malignant hypertension and the inability to concentrate urine. Of particular interest, the mRen(2).Lewis strain also demonstrates a significant gender difference in the development of hypertension. Therefore, in the present studies, we tested the hypothesis that early depletion of estrogen...
Heterozygous male and female mRen(2).Lewis rats were obtained from the Hypertension and Vascular Disease Center Transgenic colony at 5 weeks of age, and either a bilateral ovariectomy or sham surgery was performed on the female littermates. A separate group of ovariectomized (OVX)-mRen(2).Lewis was implanted with a 3-week 17β-estradiol pellet (1.0 mg pellet) subcutaneously after the ovariectomy and again 3 weeks later. These groups were killed at 11 weeks to assess both circulating and tissue RAS components as well as 17β-estradiol serum levels. Two additional groups of OVX-mRen(2).Lewis rats were either treated with the AT1 antagonist olmesartan (1 mg/kg per day OS) for 4 weeks (weeks 12 to 16) to assess the influence of RAS blockade during the period of established hypertension or used as age-matched control animals. At the end of 16 weeks, the drug was completely removed from the drinking water and the rats were monitored for an additional 7 weeks. Finally, age-matched normotensive Lewis rats (Harlan Labs) were ovariectomized, and blood pressure was determined from week 5 to week 17. Systolic blood pressure (SBP) was measured in trained rats (mean of 5 determinations per data point) before and after treatment with a Narco Biosystems device. Animals were fed a powdered rat chow (Purina Mills) to provide a daily intake of 17 and 28 mEq of sodium and potassium, respectively, per 100 g of body weight. At the end of the study, animals were euthanized by decapitation without anesthesia, and tissues were rapidly removed for analysis. Rats were maintained on a 12-hour light/dark cycle (6 AM on, 6 PM off) at constant temperature and humidity in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with protocols of the Institutional Animal Care and Use Committee of Wake Forest University School of Medicine.

The concentration of Angiotensin (Ang) II was determined in the plasma, urine, and kidneys from control and treated rats, as described by Allred et al.14 Urine was collected over a 24-hour period into 4% acetic acid and BHT. Excretion of 8-isoprostane F2 isoprostane (Cayman Chemicals) and endothelin-1 (ALPCO Diagnostics) were determined according to instructions for each assay kit. Serum estradiol concentrations were determined by RIA with a kit from Adaltis Italia S.P.A. Serum ACE activity was determined with the synthetic substrate Hip-His-Leu in the presence and absence of the ACE inhibitor lisinopril (10 μmol/L). Plasma renin concentration (PRC) was determined by addition of exogenous angiotensining (neophrectomized rat plasma) incubated at either pH 6.5 (rat renin) or pH 8.5 (mouse renin).

Total RNA was isolated from the renal cortex and medulla of each rat with TRIzol (Invitrogen) and then amplified with or without AMV reverse transcriptase, as described by Gallagher et al.1 Primer pairs were eNOS forward 5'-CTGCTGCCGAGATATCTTC-3' and reverse 5'-AAGTTAAAGGGAGGAGCGCGCA-3', yielding a 435-bp fragment; ACE forward 5'-TTGACGTGAACACCTCCCA-3' and reverse 5'-GGCTGCACTCTTGATAG-3', yielding a 421-bp fragment; and EF1α forward 5'-GGAATGTTGACAAATGCTG-3' and reverse 5'-CGTTGAAAGGCTACATGTCC-3', yielding a 347-bp fragment. Amplification conditions were 60-second denaturation at 94°C, annealing for 60 seconds at 60°C for ACE or 62°C for eNOS, and extension at 72°C for 30 cycles followed by a final extension at 72°C for 5 minutes. EF1α primers were added after 10 cycles were completed. Products were separated on 6% polyacrylamide gels, and band intensities were quantified by phosphorimager analysis. The mRNA concentration was expressed as the ratio of eNOS or ACE to the control EF1α to account for variations in the RT-PCR assay. We use EF1α because Ang II is known to regulate other “control” genes such as GADPH.

Statistical Analysis

All measurements are expressed as mean±SEM. Comparisons between the sham, ovariectomized, and treated rats were evaluated by ANOVA and the Dunnett post hoc analysis (StatMate). All other data were analyzed by the Student unpaired t test, and figures were constructed with GraphPad Prism plotting and statistical software. A probability value of <0.05 was required for statistical significance.

Results

Compared with the sham mRen(2).Lewis rats, the OVX congensics had an elevated blood pressure as early as week 6 (109±3.0 mm Hg versus 129±4.2 mm Hg) (Figure 1). Although the sham SBP plateaued by week 9 (139±2.5 mm Hg versus 141±3.9 mm Hg at week 11), the OVX-mRen(2).Lewis continued to have a higher blood pressure at week 9 (171±4.0 mm Hg) through week 11 (195±3.7 mm Hg). The estrogen replacement group also had a greater increase in blood pressure than the sham rats up to week 7 (139±2.3 mm Hg versus 115±3.9 mm Hg for Sham, P<0.01) and was similar to the OVX rats (145±3.7 mm Hg, 7 weeks). However, by week 9, the blood pressure began to decline (135±2.9 mm Hg) and was significantly lower (125±2.9 mm Hg, P<0.05) than either the Sham or OVX-REN(2).Lewis at 11 weeks. Although not shown, ovariectomy of the normotensive Lewis rats did not alter blood pressure (intact Lewis: 112±9.3 mm Hg, n=5 versus OVX-Lewis: 118±6.7 mm Hg, n=3; both groups at 12 weeks of age). Furthermore, the blood pressures in the OVX-mRen(2).Lewis rat were similar to that of the male mRen(2).Lewis at 11 weeks (205±2.9 mm Hg, n=5), suggesting that estrogen depletion at this age abolishes the gender difference in the mRen(2).Lewis rat. Finally, in the female mRen(2).Lewis rats, serum estriadiol levels at 11 weeks averaged 88±17 pg/mL for sham, <5.0 pg/mL in the OVX group, and 194±29 pg/mL with estrogen replacement.

As shown in Figure 2A, estrogen depletion in the mRen(2).Lewis increased PRC approximately 2-fold compared with either the sham or estrogen-treated groups. Measurement of
mouse PRC revealed a similar level to that of rat renin after ovariectomy and estrogen replacement (Figure 2B). Serum ACE activity was also significantly higher in the OVX-mRen(2).Lewis as compared with the other two groups, although the estrogen-treated animals tended to have the lowest ACE activity (Figure 2C), consistent with the lower blood pressure in this group (see Figure 1). Also consistent with an increased activity of both renin and ACE, the circulating levels of Ang II were elevated to a similar degree compared with intact and estrogen-treated groups (Figure 2D). We also determined the free plasma concentration of 8-isoprostane F2 \_H9251 as an index of ROS but did not observe a difference between the OVX and estrogen-treated groups (26.8±4.6 fmol/mL versus 36.5±8.2 fmol/mL, respectively).

In terms of the renal RAS, urinary excretion and cortical tissue levels of Ang II were significantly increased in the OVX-mRen(2).Lewis rats (Figures 3A and 3B). The urinary excretion of both endothelin-1 (ET-1) and 8-isoprostane F2 \_H9251 were also elevated in the OVX group in comparison to either the sham or estrogen-treated rats (Figures 3C and 3D). Consistent with the increase in serum ACE activity and renal Ang II, both cortical and medullary levels of ACE mRNA were significantly higher in the OVX group as compared with the estrogen group (Figure 3E). Finally, we assessed mRNA levels of eNOS in the OVX-mRen(2).Lewis and the estrogen replacement group. The mRNA levels for eNOS (expressed as a ratio to EF1 \_H9251) were reduced 42% [0.273±0.016 U versus 0.466±0.073 U, P<0.01, n=6 to 7] in the renal medulla of the OVX-mRen(2).Lewis as compared with the estrogen-treated group. The overall cortical expression of eNOS was significantly lower than that in the medulla; however, estrogen depletion had no effect on cortical eNOS levels [OVX: 0.123±0.013 U versus OVX+E2: 0.103±0.011 U, P>0.05, n=6 to 7].

As shown in Figure 4, oral administration of olmesartan (1 mg/kg per day) for 2 weeks to 12-week OVX-mRen(2).Lewis rats substantially reduced blood pressure and, by 4 weeks, blood pressure was not different than the untreated normotensive female Lewis rats of similar age (113±6.4 mm Hg, n=6 versus 118±7 mm Hg for Lewis, n=5). In addition, we determined the blood pressure in this group of rats after the cessation of olmesartan treatment (week 16) for 7 weeks. By the end of week 23, the systolic blood pressures in the olmesartan-treated OVX-mRen(2).Lewis were still substantially reduced as compared with either that before treatment at 12 weeks (124±4.1 mm Hg versus 201±11 mm Hg, P<0.01) or a separate group of age-matched untreated OVX-mRen(2).Lewis at 16 weeks (190±9 mm Hg, n=5) and 23 weeks (208±12 mm Hg) but not different from that of the 16-week OVX-mRen(2).Lewis group receiving olmesartan (Figure 4).

**Discussion**

In the present study, we characterized the effects of estrogen depletion on the development of blood pressure and expression of the circulating and renal components of the RAS in the female mRen(2).Lewis rat—a monogenetic strain that overexpresses the mouse renin 2 gene. Similar to other hypertensive models and the original (mRen2)-27 transgenic strain, the mRen(2).Lewis rats exhibit a significant gender...
difference in the expression of blood pressure. Indeed, the systolic blood pressure in the heterozygous female mRen(2).Lewis rat is 40 to 50 mm Hg lower than male littermates. However, in contrast to estrogen depletion in young SHR, our findings reveal a significant effect of estrogen depletion on the development and maintenance of blood pressure in the mRen(2).Lewis rats. Indeed, estrogen depletion essentially abolished the gender difference in blood pressure that normally exists between the male and female mRen(2).Lewis rats. Low-dose estrogen (estradiol) replacement reversed the course of the development of hypertension and lowered blood pressure below that of the intact mRen(2).Lewis rat. Furthermore, chronic treatment with the AT1 receptor antagonist olmesarbn normalized the blood pressure in the ovariectomized mRen(2).Lewis. These findings clearly support recent studies that also found a protective role for estrogen in the development of hypertension in the Dahl S model maintained on either a low or high salt diet and the SHR fed a phytoestrogen-free high salt diet.

Previous studies in female (mRen2)-27 rats, the founder strain for the congenic mRen(2).Lewis rats, revealed differences in the blood pressure response after ovariectomy. Bachman et al reported that ovariectomy in 16-week heterozygous (mRen2)-27 reduced blood pressure by ~20 mm Hg. The reduction in blood pressure was associated with a fall in both PRA and Ang II, as well as reduced expression of rat and mouse renin mRNA levels in the kidney. Although the effects of estrogen replacement were not determined in this study, the lower PRA after ovariectomy is consistent with a stimulatory effect of estrogen on renin. In contrast, Brosnihan et al found that ovariectomy had little effect on blood pressure in 12-week (mRen2)-27, but estrogen replacement reduced blood pressure as well as decreased ACE activity and mRNA levels of the enzyme in the lung, aorta, and kidney. In addition, the reduction in ACE was associated with a differential expression of plasma Ang peptides, reduced Ang II, and increased Ang-(1-7) that most likely reflects the ability of ACE to form Ang II and degrade Ang-(1-7). Similar to the latter study, the present results revealed increased plasma Ang II and serum ACE activity as well as increased rat PRC after ovariectomy. Moreover, estrogen replacement reversed the changes in these parameters as well as attenuated the development of hypertension. Apart from the background of the
Ang II and the AT1 receptor after estrogen depletion may be
mediated by the expression of hypertension in this model. 10 In this
study, we report that the anti-hypertensive actions of the AT1
antagonist olmesartan were significantly attenuated by estrogen
depletion. In the ovariectomized mRen(2).Lewis, the systolic
pressures were still markedly reduced (~36%) at 7 weeks after
cessation of olmesartan administration and were not different than the
blood pressures determined at the end of the 4-week treatment period (~42%).

Perspectives
The role of estrogen to influence the cardiovascular system,
particularly in a hypertensive setting, is complex. The
mRen(2).Lewis strain represents a model of monogenetic
Ang II–dependent hypertension in which estrogen depletion
has a profound influence on the development of blood
pressure, most likely through attenuating activation of the
RAS and other downstream mediators. The early influence of
estrogen in the current studies may be more relevant to the
status of estrogen in the premenopausal versus postmeno-
pausal period. Kaplan and colleagues 44 have demonstrated
that reduced levels of estrogen arising from stress in the
premenopausal period are associated with increased inci-
dence of cardiovascular disease. Thus, the early loss or
reduction of estrogen may play a more significant role in the

(mRen2)-27 transgenics (Sprague-Dawley versus Lewis),
the major difference in the present study was that ovariectomy was
performed at an earlier age before the establishment of hyper-
tension. Interestingly, ovariectomy in the SHR as early as 3
weeks does not alter the development of blood pressure or
exacerbate the hypertension. 8 Indeed, androgens are thought to
medicate the expression of hypertension in this model. 10 In this
regard, Baltatu et al 21 recently reported that the androgen
antagonist flutamide reduced blood pressure as well as attenu-
ated the development of cardiac and renal injury in female
(mRen2)-27 transgenics. The influence of androgen blockade in the
mRen(2).Lewis is currently not known. It is possible that estrogen
could well have a modulatory influence on the actions of
androgens in this model, although studies on the effects of the
testosterone antagonist in intact or estrogen depleted
mRen(2).Lewis are necessary to address this issue.

Additional evidence that estrogen may influence the RAS
and the development of hypertension in the mRen(2).Lewis are the
results that blood pressure was normalized after 4-week treat-
ment with the AT1 receptor antagonist olmesartan. Indeed,
systolic blood pressures in the olmesartan-treated OVX-
mRen(2).Lewis group were not different from that of either the
intact or OVX-Lewis rat. Estrogen reduces the expression of the
AT1 receptor in the vasculature, adrenal gland, and kidney, as
well as inhibits the AT1-receptor actions of Ang II in vascular
smooth muscle cells. 4 6 The effects of increased expression of
Ang II and the AT1 receptor after estrogen depletion may be
further enhanced by the reduced capacity of eNOS in the kidney.
Cowley and colleagues 22 showed that reduced NO production
in the renal medulla significantly influences blood pressure in the
Dahl S rats. The influence of NOS may also explain the delayed
effects of exogenous estrogen to attenuate the development of
hypertension in the OVX-mRen(2).Lewis rats. Rahimian et al 23
observed that 3 weeks of estrogen treatment was required to
restore NO activity after ovariectomy. This treatment period
corresponds to the time point for the development of blood
pressure to diverge in the present study. Studies are in progress
to determine whether the treatment of ovariectomized
mRen(2).Lewis with olmesartan restores the renal expression of
eNOS. Regarding the influence of other mediators, we do not
know whether alterations in the renal excretion of ET-1 and
isoprostanes are directly influenced by estrogen or an activated
Ang II–AT1 axis. Ang II–dependent hypertension is associated with both increased ET-1 and activated ROS. 24–28 Furthermore,
RAS blockade and estrogen attenuate expression of both sys-
tems. 28–32 However, the issue of enhanced renal ET-1 is com-
plicated by the presence of both ET-A and ET-B receptors in the
kidneys that mediate opposing actions. 33 Thus, the balance of
these two receptors may dictate whether increased ET-1 contrib-
utes to or counters the increase in blood pressure after estrogen
depletion. In addition, the effects of estrogen depletion may
encompass increased ROS in the kidney that may further
diminish the bioavailability of NO. 32,34 Additional studies to
assess whether the blockade of these two systems contributes to
the increase in blood pressure or alters renal status in the
mRen(2).Lewis rat are also in progress.

Finally, an unanticipated finding of the present study was the
persistent effect of olmesartan treatment on blood pressure once
the antagonist was withdrawn. In the ovariectomized
mRen(2).Lewis, the systolic pressures were still markedly reduced
(~36%) at 7 weeks after cessation of olmesartan admin-
istration and were not different than the blood pressures deter-
mined at the end of the 4-week treatment period (~42%).
Previous studies, all performed in the SHR, clearly showed that
RAS blockade with either an ACE inhibitor or ARB exhibits
similar effects, provided that treatment is initiated at an early age
(3 to 4 weeks) before the development of hypertension. 35–38
Furthermore, the long-lasting effects are generally not shared by
other anti-hypertensive drugs despite a similar reduction in
pressure during the treatment period. 39–41 In contrast, studies in
the Milan and Lyon hypertensive rats revealed that RAS block-
ade had no persistent effect on blood pressure despite marked
improvements in indexes of vascular and renal injury. 42–43 To
our knowledge, the present study is the first to demonstrate a
substantial effect on blood pressure after cessation of AT1
receptor blockade in adult females with established hypertension
and in a hypertensive model other than the SHR. 38 Future studies
are necessary to ascertain the mechanism and the duration of the
persistent effects of olmesartan in the mRen(2).Lewis rat. In this
regard, it will also be of interest to determine whether cessation
of estrogen treatment in the ovariectomized mRen(2).Lewis rat
also exhibits a persistent effect on blood pressure similar to that
of the AT1 antagonist.

Figure 4. Effect of AT1 antagonist olmesartan on SBP in ovari-
ectomized (OVX) mRen(2).Lewis rat. Olmesartan (OLM, 1 mg/kg
per day) was administered for 4 weeks to OVX-mRen(2).Lewis
rats (12 weeks) with established hypertension. At week 16, drug
treatment ceased and blood pressure was assessed for an
additional 7 weeks (23 weeks of age). Data are mean±SEM;
**P<0.01 vs 12-week OVX rats, n=6.
setting and progression of cardiovascular disease than estrogen loss in the postmenopausal state.45

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References

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