Estrogen Regulation of Angiotensin-Converting Enzyme mRNA

Patricia E. Gallagher, Ping Li, John R. Lenhart, Mark C. Chappell, K. Bridget Brosnihan

Abstract—Estrogen replacement therapy is cardioprotective in postmenopausal women; however, the precise molecular mechanisms for this modulation are not fully elucidated. We previously showed that chronic estrogen replacement therapy reduced angiotensin-converting enzyme (ACE) activity in tissue extracts and serum with an associated reduction in plasma angiotensin II. A reverse transcriptase–polymerase chain reaction assay was developed to determine whether estrogen treatment regulates tissue ACE mRNA concentration. Total RNA was isolated from kidney cortex, kidney medulla, lung, and aorta of ovariectomized Sprague-Dawley rats after 21 days of chronic 17β-estradiol replacement therapy (5 mg pellet per rat SC) or placebo. A marked decrease in densitometric intensity ratios of amplified ACE cDNA to elongation factor-1α control cDNA was observed in all tissues from placebo-treated rats compared with the estradiol-treated rats (renal cortex: 0.29±0.04 versus 0.14±0.02; renal medulla: 0.37±0.04 versus 0.24±0.03; lung: 4.49±0.37 versus 2.49±0.59; and aorta: 0.41±0.04 versus 0.29±0.02; all P<0.05). A comparable reduction in ACE activity was detected in tissue extracts from kidney cortex, kidney medulla, and lung of hormone-treated animals. Incubation of purified rat lung ACE with 1 or 10 μmol/L 17β-estradiol had no effect on enzyme activity. These results suggest that estrogen treatment regulates tissue ACE activity by reducing ACE mRNA concentrations. Thus, the beneficial cardiovascular effects of estrogen may be mediated in part by downregulation of ACE with a consequent reduction in the circulating levels of the vasoconstrictor angiotensin II, a decrease in the metabolism of the vasodilator bradykinin, and an increase in the production of the vasorelaxant angiotensin-(1–7). (Hypertension. 1999;33[part II]:323-328.)

Key Words: angiotensin-converting enzyme ■ estrogen ■ gene regulation ■ hormone replacement ■ reverse transcriptase–polymerase chain reaction

Gender differences in the development of cardiovascular disease are documented in both animal and human studies. Cardiovascular disease is more prevalent in men than in premenopausal women, but the incidence increases sharply in perimenopausal women.1,2 Furthermore, estrogen replacement therapy reduces the risk of coronary artery disease in postmenopausal women, while young, bilaterally oophorectomized women have an increased occurrence of coronary disease.3-5 Thus, circulating endogenous estrogens are proposed to exert a cardioprotective effect, promoting the widespread recommendation for estrogen use in postmenopausal replacement therapy. The protective effect of estrogen is mediated, in large part, by modulating lipoprotein metabolism.3,4,6 Estrogens increased HDL cholesterol, decreased plasma LDL cholesterol, and attenuated oxidized LDL production.7-10 These actions effectively diminish accumulation of proatherogenic LDL in the arterial wall and reduce coronary artery atherosclerosis. While effects on lipid profiles account for a significant portion of the protective action of estrogen, differences in lipid levels do not entirely explain the influence of gender on the development of cardiovascular disease.

We previously showed that angiotensin-converting enzyme (ACE) activity is reduced in 2 models of postmenopausal chronic hormone replacement. ACE, a zinc-containing dipeptidyl carboxypeptidase (EC 3.4.15.1), is a key enzyme of the renin angiotensin system that plays an important role in the regulation of cardiac function and blood pressure.11-13 ACE is responsible for the local conversion of angiotensin I (Ang I) to the potent vasoconstrictor angiotensin II (Ang II). Ang II has a wide variety of effects on target organs of the cardiovascular system, including systemic and coronary artery vasconstriction and stimulation of vascular smooth muscle cell growth.14 Conversely, bradykinin, a naturally occurring nonapeptide, is locally degraded by ACE.11-13 Bradykinin acts as a potent vasodilator of peripheral arteries by a variety of mechanisms, including the release of nitric oxide, prostaglandins, and endothelial-derived hyperpolarizing factor.15,16 In addition, angiotensin-(1–7) [Ang-(1–7)], which dilates coronary arteries through the release of nitric oxide or the potentiation of bradykinin effectiveness,17,18 is degraded and inactivated by ACE.19 Thus, a reduction in ACE activity leads not only to an attenuation of Ang II production and a decrease

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From The Hypertension and Vascular Disease Center, Division of Surgical Sciences, Wake Forest University School of Medicine, Winston-Salem, NC. Correspondence to Patricia E. Gallagher, The Hypertension and Vascular Disease Center, Medical Center Blvd, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1032. E-mail pgallagh@wfu.edu
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in bradykinin degradation but also to an increase in the vasodilator Ang-(1–7), an active metabolite of Ang I. ACE inhibitors are clinically effective in the treatment of hypertension and associated end-organ disease.\(^\text{12,13}\) We observed an attenuation of ACE activity in surgically induced postmenopausal cynomolgus monkeys treated with conjugated equine estrogen (Premarin) for 30 months.\(^\text{20}\) This reduction in ACE activity after estrogen treatment was associated with a significant increase in plasma Ang I and hyperreninemia but with a reduced formation of Ang II. Furthermore, estradiol replacement therapy decreased serum ACE activity in both ovariectomized normotensive and hypertensive (mRen2)\(^\text{27}\) transgenic rats, which contain an inserted mouse renin gene.\(^\text{21}\) Kidney and aorta tissue extracts from the hormone-treated transgenic rats also exhibited a significant reduction in enzyme activity. The effects of estrogen were associated with a moderation of the hypertension in the transgenic rats and a lowering of blood pressure in the normotensive animals. These results suggest that estrogen may mediate its cardioprotective effect in part by modulating ACE activity. This report is the first to show that estrogen treatment regulates the activity of ACE in tissues by reducing ACE mRNA concentrations.

**Methods**

**Animal Treatment**

Bilateral oophorectomy was performed on 12-week-old Sprague-Dawley rats (220 to 225 g) under general anesthesia with ketamine (30 mg/kg IM) and xylazine (5 mg/kg IM). The ovariectomized rats were randomly divided into 2 groups, and pellets containing either 17\(^\beta\)-estradiol (5 mg per rat with a 3-week release; Innovative Research of America)\(^\text{22}\) or placebo were implanted in the subcutaneous tissue. After recovery, the rats were allowed free access to water and were fed standard rat chow. The animals were housed in individual cages and maintained at 22°C with 12 hours of light. After 18 to 20 days, the rats were killed by decapitation, and trunk blood was collected into prechilled tubes for determination of serum 17\(^\beta\)-estradiol concentration and serum ACE activity. In addition, the kidneys, aorta, and lungs were harvested and dissected free of connective tissue on ice, and the kidney cortex and medulla were separated. The tissues were quick-frozen on dry ice and stored at \(-80^\circ\text{C}\) until analyzed for ACE mRNA concentration and ACE activity.

**17\(^\beta\)-Estradiol Measurement**

Plasma estradiol concentrations were determined by radioimmunoassay with the use of a commercially available kit (Polymedco, Inc.).

**Reverse Transcriptase–Polymerase Chain Reaction Assay**

Total RNA was isolated from the kidney, lung, and aorta of each rat with the TRIZOL reagent (GIBCO BRL Products), as directed by the manufacturer. The RNAs were processed and analyzed separately. The RNA concentration was quantified by UV spectroscopy, and any degradation was assessed by ethidium bromide staining intensity of 28S and 18S ribosomal RNA after agarose gel electrophoresis. The RNA was incubated with RNQ DNase (Promega Corp) to eliminate any residual DNA that would amplify during the polymerase chain reaction (PCR). Approximately 250 ng of total RNA was incubated with or without avian myeloblastosis virus (AMV) reverse transcriptase (RT) (Promega Corp) in a mixture containing deoxynucleotides, random hexamers, and RNase inhibitor in RT buffer.\(^\text{23}\) Heating the mixture for 5 minutes at 95°C terminated the reaction.

For amplification of the resulting cDNA, 1 \(\mu\)mol/L gene-specific primers, 0.2 mmol/L deoxynucleotides, 1.5 mmol/L MgCl\(_2\), and 1.5 U Taq polymerase were added to 3 \(\mu\)L of each RNA sample in a final volume of 50 \(\mu\)L. Primer sequences were based on the sequences of the cloned ACE gene\(^\text{24,25}\) and elongation factor-1\(^\alpha\) (EF1\(^\alpha\)) gene.\(^\text{26}\) Primer sets were as follows: ACE forward primer 5\text{'-}\text{CAGCTTCATCATCCGTTTC}-3\text{' and reverse primer 5\text{'-}\text{CTAGGAAAGCAGCCACCC}-3\text{'}, yielding an amplification product of 406 base pairs, and EF1\(^\alpha\) forward primer 5\text{'-}\text{GAATGGAACGACATGCTG}-3\text{' and reverse primer 5\text{'-}\text{GGTGAAGCCTACATGTC}-3\text{'}, resulting in a 347–base pair product. Any variations in reverse transcription or amplification efficiency, RNA degradation, pipetting, or sample loading were detected by the cotranscription and amplification of the positive control mRNA, EF1\(^\alpha\), since ACE mRNA concentration was quantified as a ratio of the amount of target cDNA/EF1\(^\alpha\) cDNA. The abundant, housekeeping transcript EF1\(^\alpha\) was used as a positive control mRNA rather than GADPH, a conventional standard, because previous studies showed that GADPH mRNA is upregulated by Ang II.\(^\text{27}\) Approximately 5 \(\mu\)Ci \[^{3}\text{H}\]dCTP was also included in the reaction for subsequent quantification of the amplification products. Amplification conditions were as follows: denaturation at 94°C for 60 seconds; annealing at 62°C for 60 seconds; and elongation at 72°C for 60 seconds for 28 cycles, with a final elongation step at 72°C for 7 minutes. Primers for the control EF1\(^\alpha\) sequence were added after 5 amplification cycles were completed. After PCR, the amplification products were separated on a 6% polyacrylamide gel and visualized by autoradiography. Bands intensities were quantified with the use of computerized densitometry (Micro Computer Imaging Device, Imaging Research Inc.).

A DNA sequence of amplified products was obtained during the development of the ACE RT-PCR assay and each time a new primer set was purchased. The sequence analysis as well as the molecular size of the amplified products confirmed accurate RT-PCR of the mRNAs of interest. In the development of the ACE RT-PCR assay, optimum amplification conditions and reagent concentrations were carefully determined. In addition, the cycle number was optimized for linear amplification, since a loss of linearity occurs with increased cycle number because of reagent depletion and reduced primer-annealing efficiency. False-positive signals produced by the introduction of contaminating DNA into this sensitive assay were detected in a complementary RT minus reaction.

**Serum ACE Activity Assay**

Serum ACE activity was measured with a commercially available kit (Hycor). Duplicate aliquots of rat serum were incubated with the tripeptide substrate \[^{[3}\text{H}\]hippuryl-glycyl-glycine for 60 minutes at 37°C. The reaction mixture was acidified, and the \[^{[3}\text{H}\]hippuric acid released by the enzyme was extracted into ethyl acetate with quantified by liquid scintillation spectrometry.

**Tissue ACE Activity Assay**

Approximately 250 to 300 mg (wet weight) of kidney cortex, kidney medulla, or lung tissue was minced and homogenized with the use of a Teflon-glass homogenizer in 3.0 mL of extraction buffer (10 mmol/L HEPES, pH 7.4, containing 125 mmol/L NaCl). The cellular membranes were isolated by centrifugation at 12 000g for 20 minutes at 4°C and rehomogenized in 3.0 mL of extraction buffer. The membrane fraction was isolated by a second centrifugation, and the resultant pellet was resuspended in 3.0 mL of extraction buffer by homogenization. Protein content of the membrane fraction was determined by the method of Lowry et al\(^\text{26}\) using bovine serum albumin as a standard. Aliquots of the resuspended membranes were incubated with 30 mmol/L Hip-His-Leu for 15 minutes at 37°C. The ACE inhibitor lisinopril (1 \(\mu\)mol/L) was added to a second set of aliquots as a control for ACE specificity. Enzyme activity was quantified by fluorometric measurement of hippurate cleavage from the substrate and compared with standards.
17β-Estradiol Treatment of Purified ACE
ACE was isolated from a membrane fraction of rat lung with the use of a lisinopril-coupled affinity column, as previously described. A single band corresponding to the 170-kDa somatic ACE was obtained after polyacrylamide gel electrophoresis, indicating the high degree of purity of the enzyme preparation.

Aliquots of the purified enzyme were incubated with 30 mmol/L Hip-His-Leu and 1 or 10 μmol/L 17β-estradiol for 15 minutes at 37°C. A mixture without 17β-estradiol served as a control. The ACE inhibitor lisinopril (1 μmol/L) was added to a second set of aliquots as a control for ACE specificity. Enzyme activity was quantified by fluorometric measurement of hippurate cleavage from the substrate and compared with standards.

Statistical Analysis
Data are expressed as mean±SEM. Differences between groups were compared with Student’s t test for unpaired data. Statistical significance was accepted with probability value <0.05.

Results

17β-Estradiol Concentration in Ovariectomized Rats
A radioimmunoassay was used to verify an increase in plasma 17β-estradiol from ovariectomized Sprague-Dawley rats treated for 18 to 20 days with implanted pellets continuously releasing hormone compared with control animals. The concentrations averaged 894±114 pg/mL in 17β-estradiol–treated rats (n=8) and <15 pg/mL in placebo-treated animals (n=8; P<0.05 compared with treated rats).

Effect of Hormone Replacement on ACE mRNA Concentration
Total RNA was isolated from the kidney cortex, kidney medulla, lung, or aorta of Sprague-Dawley rats after 3 weeks of 17β-estradiol treatment or placebo to determine whether hormone replacement regulates ACE mRNA concentration. An autoradiogram typical of a RT-PCR determination for kidney cortex and medulla is shown in Figure 1A. A marked decrease in band intensities corresponding to ACE mRNA was observed in both kidney cortex and kidney medulla samples from hormone-treated rats compared with controls. Autoradiograms of RT-PCR analysis of lung or aorta mRNA isolated from rats after 17β-estradiol treatment or from placebo controls showed similar patterns (Figures 2A and 3A, respectively).

Densitometric intensity ratios of amplified ACE cDNA to EF1α control cDNA significantly decreased in the kidney cortex (0.29±0.04 versus 0.14±0.02; Figure 1B) and kidney medulla (0.37±0.04 versus 0.24±0.03; Figure 1B) of controls compared with the estradiol-treated rat samples. Three weeks of hormone replacement therapy also induced a 1.8-fold decrease in ACE mRNA concentrations in the rat lung (4.9±0.37 versus 2.49±0.59; Figure 2B) from hormone-treated rats compared with controls. A similar reduction in ACE transcript concentration (0.41±0.04 versus 0.29±0.02; Figure 3B) was observed in samples isolated from the aortas of estradiol-treated rats.

Effect of Hormone Replacement on Serum and Tissue ACE Activity
ACE activity was measured in tissue homogenates of placebo- and estradiol-treated rats to verify that reduced ACE mRNA correlated with decreased enzyme activity. As shown in Figure 4, ACE activity was attenuated in tissue extracts from kidney cortex (2.01±0.28 versus 1.24±0.16 nmol/mg per minute), kidney medulla (1.52±0.23 versus 0.86±0.13 nmol/mg per minute), and lung (58.31±6.58 versus 34.62±4.25 nmol/mg per minute) after 3 weeks of 17β-estradiol treatment compared with placebo controls. Serum
ACE activity decreased from 98.8±6.6 nmol/mL per minute (n=8) to 71.0±5.7 nmol/mL per minute (n=8; P<0.05) with hormone replacement. In contrast, incubation of purified rat lung ACE with 1 or 10 μmol/L 17β-estradiol did not reduce the enzyme activity (data not shown).

Discussion

Our studies demonstrate for the first time that estrogen treatment modulates ACE mRNA concentration in rat kidney, lung, and aorta, with a resultant attenuation of ACE activity. The reduction in ACE activity was not due to a direct interaction of estrogen with the enzyme. These results suggest that the cardioprotective properties of estrogen are due in part to the diminished ACE activity in vivo through inhibition of ACE mRNA synthesis. These findings support further studies of chronic hormone replacement therapy for therapeutic intervention.

We previously showed that ACE activity is reduced in 2 models of postmenopausal chronic hormone replacement: ovariectomized monkeys treated with conjugated equine estrogen (Premarin) for 30 months20 and ovariectomized normal and transgenic rats on 3 weeks of estradiol replacement therapy.21 In our previous publication,21 we used an estrogen dose (1.5 mg/rat for a 3-week release) that raised the levels of circulating estrogen to 150 to 190 pg/mL. This 17β-estradiol concentration was characterized as a physiological dose during the estrous or menstrual cycle29,30 or after hormone replacement.31 This dose reduced the levels of plasma ACE in both Sprague-Dawley and transgenic rats by 40% to 50%.21 In the present studies we used a higher dose of estrogen (5 mg/pellet), which raised the circulating 17β-estradiol slightly more than 4-fold (to 894 pg/mL) and decreased the circulating levels of ACE activity 30%. Thus, both the higher and lower doses of 17β-estradiol had nearly equivalent effects on circulating ACE activity.

Studies by other investigators also showed that ACE activity is regulated by estrogen. Seltzer et al32 demonstrated that estrogen administration to ovariectomized rats reduced ACE activity in the anterior pituitary. No change in pituitary or circulating ACE activity was detected with the normal estrus cycle, suggesting that attenuated ACE activity occurs only after chronic exposure to estrogen at doses in excess of endogenous concentrations. Furthermore, pituitary ACE from ovariectomized rats treated with estradiol showed a decreased V_max compared with controls, indicating that estrogen may reduce ACE production. Female mice homozygous for the ACE gene have 60% lower plasma ACE activity compared with males, indicating a sexual dimorphism.33 Serum ACE activity was also reduced in the first and second trimesters in normotensive pregnant women with elevated plasma estrogen compared with nonpregnant subjects.34,35 In addition, postmenopausal women on estrogen hormone replacement showed a slight but significant lower serum ACE activity compared with those without hormone replacement.36 Because androgens have no effect on somatic ACE activity,37 these findings suggest that the sexual dimorphism for ACE activity between males and females may be due in part to estrogen-induced reduction of ACE mRNA and ACE activity.

Estrogens, like all members of the steroid family of hormones, regulate cellular processes through specific hormone receptor–mediated mechanisms.38 The estrogen-receptor complex regulates transcription of a variety of genes by binding to estrogen response elements and affecting RNA polymerase activity. Such directly activated genes are referred to as primary response genes. Many of the estrogen-
regulated, primary response genes encode transcription factors. The estrogen-receptor complex also mediates gene transcription independent of its hormone response element. By this mechanism, the estrogen-receptor complex interacts directly with transcription factors, forming a multiprotein complex that binds to specific gene response elements. For example, the estrogen-receptor complex will bind with a Fos and Jun heterodimer and affect gene transcription at an API site. 39–41 Thus, estrogen mediates its regulatory effects by multiple, cascading mechanisms.

No estrogen response element (5′-GGTCANNTGACCC-3′) was reported in the 5′ flanking region of the ACE coding sequence; however, the ACE promoter does contain a consensus API site in the 300–base pair region upstream from the start site. 42–45 It is possible that the ACE mRNA regulation by estrogen treatment occurs through a receptor-mediated complex interaction with the Fos-Jun heterodimer at an API site. Estrogen also binds to imperfect estrogen response elements, albeit with lower affinity, by forming heterodimers with nonhormone proteins or through other protein–protein interactions. Thus, additional analyses of the regulatory regions of the ACE gene are needed to elucidate the precise molecular mechanism of the regulation of ACE mRNA by estrogen.

In summary, it is known that estrogen causes overexpression of both tissue and circulating concentrations of angiotensinogen 46,47 and renin. 48–51 Conversely, the present study shows that estrogen treatment reduces ACE mRNA and ACE activity. These results, in conjunction with previous studies from our laboratory, demonstrate that the reduction in ACE mRNA and ACE activity is associated with a decrease in plasma Ang II, an increase in the vasodilator peptide Ang-(1–7), and an attenuation in blood pressure. 20,21 Thus, estrogen replacement therapy, by acting at a later step in the renin-angiotensin system cascade, may contribute to enhanced cardiovascular protection by downregulating ACE mRNA concentrations, thereby reducing ACE activity. This would cause an attenuation in the production of the vasoconstrictor Ang II and an increase in the vasorelaxants bradykinin and Ang-(1–7), all of which produce favorable cardiovascular effects.

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