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Relationship Between the Angiotensin-Converting Enzyme Genotype and the Forearm Vasodilator Response to Estrogen Replacement Therapy in Postmenopausal Women

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OBJECTIVES We sought to evaluate the relationship between the angiotensin-converting enzyme (ACE) genotype and the change in forearm vasoreactivity in response to a three-month course of oral estrogen in postmenopausal women.

BACKGROUND The ACE genotype is a known predictor of the response to an ACE inhibitor drug; however, it is not clear whether it can modify the effect of estrogen replacement therapy (ERT) on endothelial function in postmenopausal women.

METHODS Fifty-five postmenopausal women received 0.625 mg of conjugated equine estrogen daily for three months. Forearm blood flow (FBF) was measured by strain-gauge plethysmography.

RESULTS Twenty-one, 25 and 9 patients had the insertion/deletion (ID), II and DD genotypes, respectively. Plasma ACE activity was significantly higher at baseline in patients with either the DD or ID genotype than in those with the II genotype ($p < 0.05$). A significant decrease in plasma ACE activity with ERT was seen in the ID and II genotypes ($p < 0.05$), but not in the DD genotype. There were no significant differences in the FBF responses to reactive hyperemia at baseline between the three groups. Estrogen replacement therapy did not alter the FBF response to reactive hyperemia in the DD genotype ($4.0 \pm 1.3\%$), although ERT significantly increased the FBF response in the ID and II genotypes ($32.6 \pm 7.5\%$ and $30.6 \pm 6.5\%$, respectively; $p < 0.05$). Forearm blood flow after administration of sublingual nitroglycerin did not change over three months in any of the three groups.

CONCLUSIONS These findings suggest that the effect of ERT in postmenopausal women on forearm endothelial function may be determined in part by the genotype of the ACE gene. (J Am Coll Cardiol 2001;37:1529–35) © 2001 by the American College of Cardiology

Premenopausal women have a lower risk of coronary heart disease than men, but the risk rapidly rises in women after menopause (1). Estrogen replacement therapy (ERT) in postmenopausal women has been associated with a reduction in cardiovascular events in some but not all studies (2–5). Estrogen exerts many protective effects on the cardiovascular system, including a lipid-lowering effect (6), an antioxidant effect (7), inhibition of fibrosis (8) and a vasodilating effect (9,10). Some studies have shown that the short- and long-term administration of estrogen increases forearm reactive hyperemia in postmenopausal women (9–12). Reactive hyperemia in the peripheral arteries is mediated by endothelial-derived nitric oxide (NO) (13).

Through its alpha-type receptor, estrogen may directly upregulate endothelial nitric oxide synthase (eNOS) gene expression (14,15). The lipid-lowering and anti-oxidant effects of estrogen may also improve endothelial-vascular function overall and diminish vascular disease, thereby increasing vascular NO production (16,17). However, the mechanism by which estrogen improves endothelial function is thought to be complex and multifactorial.

The renin-angiotensin system is reportedly suppressed by hormone therapy in postmenopausal women, with no increase in blood pressure (18,19). A decline in angiotensin-converting enzyme (ACE) activity may be one of the factors that protect against cardiovascular disease. The ACE inhibitors have been shown, in some clinical studies, to improve endothelial function (20,21). In addition, ACE inhibition induces the accumulation of bradykinin through the inhibition of kininase II. Bradykinin causes NO to be released from endothelial cells (22,23).

An insertion/deletion (ID) polymorphism of the ACE gene, which has been mapped to human chromosome 17q23, has been reported to be involved in various cardiovascular diseases, such as myocardial infarction (24), left ventricular remodeling after myocardial infarction (25) and complications of diabetes (26). Some studies (27,28) have suggested that the endothelial effects of ACE inhibitors differ according to this gene's polymorphism.

However, it is not clear whether the ACE genotype can modify the response to ERT on endothelial function in postmenopausal women. The present study determined the relationship between the ACE genotype and the change in

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Abbreviations and Acronyms

ACE	= angiotensin-converting enzyme
ANOVA	= analysis of variance
eNOS	= endothelial nitric oxide synthase
ERT	= estrogen replacement therapy
FBF	= forearm blood flow
HDL	= high density lipoprotein
ID	= insertion/deletion
LDL	= low density lipoprotein
NO	= nitric oxide
PCR	= polymerase chain reaction

forearm vasoreactivity over a three-month course of oral ERT in postmenopausal women.

METHODS

Subjects. A total of 55 postmenopausal Japanese women (age 48 to 57 years) were enrolled in the study. Each subject had experienced natural menopause for at least one year, but not longer than five years. Each woman was of normal weight, with a body mass index $\leq$25 kg/m^2. Menopausal status was confirmed by a serum follicle-stimulating hormone concentration >40 mIU/ml and a serum estradiol concentration <20 pg/ml. Excluded from the study were patients with hypertension, diabetes, a cigarette smoking habit, clinical manifestations of atherosclerosis (e.g., coronary artery disease, peripheral artery disease, cerebrovascular disease), venous thromboembolism, liver disorders, unexplained vaginal bleeding and a personal or family history of breast cancer. Before being enrolled in the study, each subject underwent a physical examination, including a gynecologic evaluation and mammography. None had received ERT, other steroid hormones or any medication known to affect blood pressure or lipoprotein metabolism.

Protocol. All subjects received conjugated equine estrogen (Premarin; Wyeth-Ayerst Laboratories, Philadelphia, Pennsylvania), 0.625 mg daily each morning for three months. All participants were followed for three months. Each subject was requested to avoid making any changes in lifestyle or dietary habits during the study. The Ethics Committee of the Department of Obstetrics and Gynecology of Hiroshima University approved the study protocol. Written, informed consent for participation was obtained from each subject.

The vasodilator response to reactive hyperemia, an index of endothelium-dependent vasodilation, and to sublingual administration of nitroglycerin, an index of endothelium-independent vasodilation, was evaluated in each subject at baseline and again after three months. This evaluation began at 8:30 AM. Each subject had fasted the previous night for at least 12 h. Before fasting, they rested in the supine position in a quiet, air-conditioned room (constant temperature 22 to 25°C). After the subject had rested for 30 min in that position, the basal forearm blood flow (FBF) was measured as described subsequently. Next, the effects of reactive hyperemia and sublingual administration of nitroglycerin on FBF were evaluated by inflating a cuff over the left upper arm to 280 mm Hg for 5 min. After the cuff occlusion was released, FBF was measured for 3 min. Next, a nitroglycerin tablet (0.3 mg) (Nihonkayaku Co., Tokyo, Japan) was administered sublingually, and FBF was again measured for 3 min. In the preliminary study, we confirmed the reproducibility of the FBF responses to reactive hyperemia and sublingual NTG on two separate occasions in 28 healthy men (mean age 27 $\pm$ 5 years). The coefficients of variation were 4.3% and 2.8%, respectively.

The baseline fasting serum concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, creatinine, glucose, electrolytes, nitrite/nitrate and ACE activity were obtained after a 30-min period of rest. The body weight, blood pressure and heart rate were determined at baseline and again after three months of treatment.

Measurement of FBF. Forearm blood flow was measured with a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D. E. Hokanson, Inc., Issaquah, Washington), as previously described (29,30). The intra-observer coefficient of variation was 3.0 $\pm$ 1.6%. Four plethysmographic measurements were averaged to obtain FBF at baseline, during reactive hyperemia and after the administration of sublingual nitroglycerin.

Deoxyribonucleic acid studies. Genomic deoxyribonucleic acid (DNA) was isolated from peripheral leukocytes. The genotype of the ACE gene was determined by the polymerase chain reaction (PCR), according to the method of Rigat et al. (31). The PCR analysis was performed with 50 μl of the reaction solution containing 50 pmol/liter of each primer, 1.5 mmol/liter of $MgCl_2$, 50 mmol/liter of KCl, 10 mmol/liter of tris-HCl (pH 8.3), 200 μmol/liter of each deoxynucleoside triphosphate and 2.5 U of *Taq* DNA polymerase (Takara Shuzo Co., Kyoto, Japan).

Analytical methods. Samples of venous blood were placed in polystyrene tubes containing sodium EDTA (1 mg/ml). The EDTA-containing tubes were immediately chilled in an ice bath. Next, the plasma was separated by centrifugation at 3,100 rpm at 4°C for 10 min. Serum was separated at 1,000 rpm at room temperature for 10 min. Samples were stored at −80°C until assayed. Routine chemical methods were used to determine the serum concentrations of total cholesterol, HDL cholesterol, triglycerides, creatinine, glucose and electrolytes. The serum concentration of low density lipoprotein (LDL) cholesterol was determined by Freidewald's method (32). Serum concentrations of follicle-stimulating hormone and estradiol were analyzed by using a commercially available radioimmunoassay kit (Boehringer, Mannheim, Germany).

Nitrite/nitrate concentrations were measured with an auto-analyzer (flow injection analyzer, TCI-NOX1000, Tokyo Kasei Kogyo, Tokyo, Japan), which uses a protocol based on the Griess reaction (33).

The ACE activity (IU/liter at 37°C) was measured with

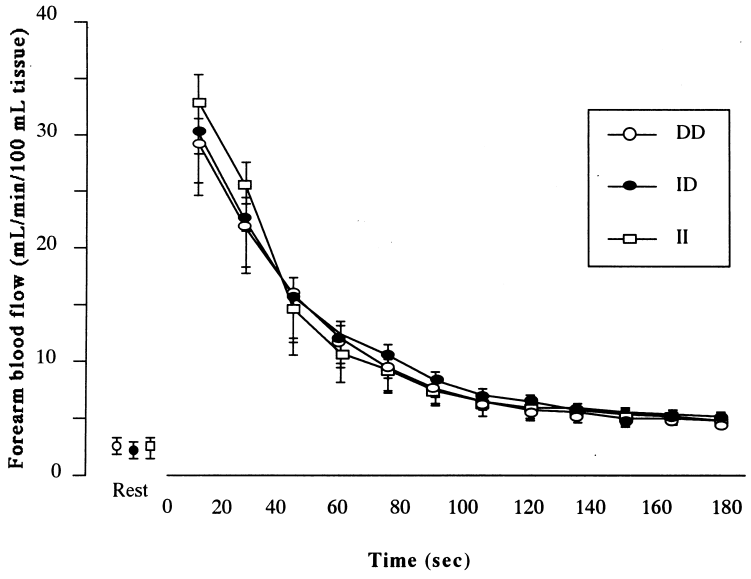

Figure 1. Forearm blood flow at rest and during reactive hyperemia in the DD (n = 9), ID (n = 21) and II (n = 25) angiotensin converting enzyne genotype groups at baseline. There was no significant difference in reactive hyperemia between the three groups at baseline.

ACE color (Fujirebio Co., Ltd., Tokyo, Japan). Plasma renin activity was measured by a radioimmunoassay kit.

Statistical analysis. The results are presented as the mean value ± SE. One-way analysis of variance (ANOVA) was used to compare the baseline clinical characteristics of the three ACE genotypes. The comparison of the time-course curves of FBF during reactive hyperemia at baseline between the three ACE genotypes was done by using two-way ANOVA for repeated measures on one factor, followed by the Bonferroni correction for multiple, paired comparisons. The repeated factor was time of reactive hyperemia, and the nonrepeated factor was one group versus the other group (Fig. 1). Comparisons between the treatment groups, with respect to changes in variables, were performed with adjusted mean values by analysis of co-variance, using the baseline data as covariates. Comparisons of variables, including maximal FBF response to nitroglycerin (Fig. 2), were carried out by one-way ANOVA, followed by the Bonferroni correction. Percent changes of the maximal FBF response to reactive hyperemia were compared between the three ACE genotypes by using one-way ANOVA (Fig. 3). A level of $p < 0.05$ was accepted as statistically significant. Data were processed using the Software package Statview IV (Brainpower) or Super ANOVA (Abacus Concepts, Berkeley, California).

RESULTS

Effects of ERT on baseline clinical variables. The ACE genotype distribution and clinical variables of the 55 postmenopausal women at baseline and again after three months are summarized in Table 1. Among the 55 patients, 9, 21 and 25 had the DD, ID and II genotypes, respectively. Age, body weight, years since menopause, blood pressure, heart rate and lipid profile were similar between the three groups. Twelve weeks of ERT significantly reduced the serum

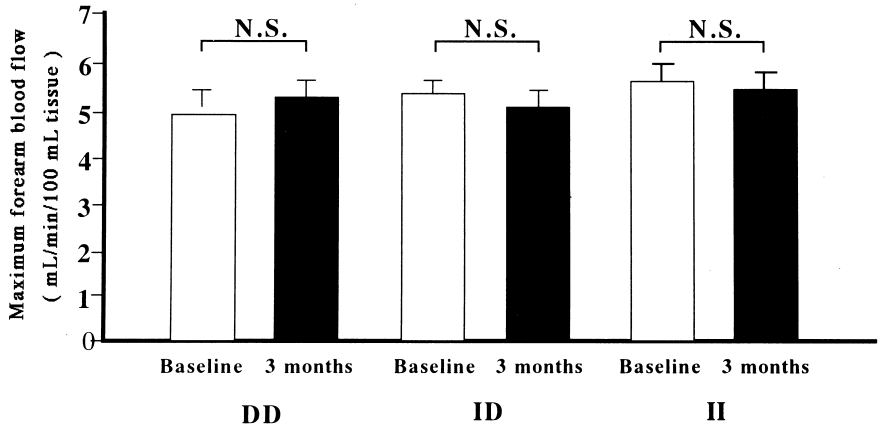

Figure 2. Maximal forearm blood flow (FBF) after sublingual administration of nitroglycerin in the DD (n = 9), ID (n = 21) and II (n = 25) ACE genotype groups at baseline and after three months of estrogen replacement therapy (ERT). Changes in FBF similar to those seen before and after ERT were observed in the three groups after the administration of sublingual nitroglycerin. Results are presented as the mean value ± SE. N.S. = not significant.

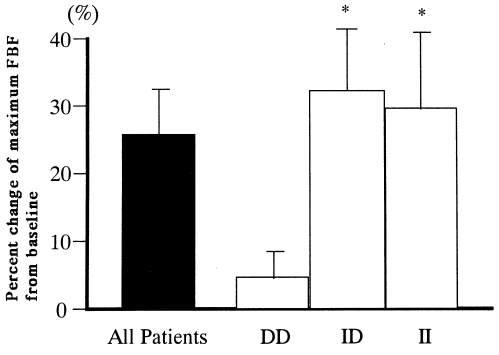

Figure 3. Absolute changes in percent maximal forearm blood flow (FBF) during reactive hyperemia after three months of estrogen replacement therapy, according to the ACE genotype. A significant increase in the maximal FBF during reactive hyperemia was seen only in the ID and II genotype groups (*$p < 0.05$). The results are presented as the mean value ± SE.

concentrations of total cholesterol and LDL cholesterol ($p < 0.05$) and increased the concentration of HDL cholesterol ($p < 0.05$), as compared with the baseline values, in all three ACE genotype groups. These changes were similar in the three groups. The baseline serum estradiol concentration in the three groups remained within the menopausal range. The serum estradiol concentration after three months of ERT was significantly higher than that at baseline ($p < 0.01$) in the three groups. The body weight, blood pressure and heart rate remained unchanged in the three groups at three months. No abnormal endometrial histologic findings (e.g., hyperplasia or carcinoma) were noted during the study.

Effects of ERT on ACE activity or nitrite/nitrate concentration and ACE genotype. The plasma ACE activity was significantly higher in the patients with either the DD or ID genotype than in those with the II genotype ($p < 0.05$). A significant decrease in plasma ACE activity with ERT was seen in the ID and II genotypes (ID: from 13.9 ± 1.0 to 12.2 ± 0.9 IU/liter at 37°C, $p < 0.01$; II: from 11.0 ± 0.4 to 9.8 ± 0.6 IU/liter at 37°C, $p < 0.01$), but not in the DD genotype (from 15.0 ± 0.9 to 14.4 ± 1.0 IU/liter at 37°C). The serum nitrite/nitrate concentrations were similar in the three groups at baseline. A significant increase in the nitrite/nitrate concentration with ERT was also seen in the ID and II genotypes (ID: from 29.3 ± 2.3 to 39.2 ± 4.1 μmol/liter, $p < 0.05$; ID: from 28.2 ± 3.7 to 36.1 ± 4.6 μmol/liter, $p < 0.05$), but not in the DD genotype (from 26.0 ± 2.0 to 30.3 ± 3.6 μmol/liter) (Table 1).

Effect of ERT on endothelial function and ACE genotype. There were no significant differences in the FBF responses to reactive hyperemia between the three groups at baseline (Fig. 1). The changes in FBF after sublingual administration of nitroglycerin were similar between the three groups at baseline versus after the three-month period of observation (Fig. 2). In response to ERT, there was no increase in the FBF responses to reactive hyperemia noted in the DD genotype group. A significant increase in the FBF responses to reactive hyperemia after ERT was restricted to those with the ID (mean percent change 32.6 ± 7.5%, $p < 0.05$) and II genotypes (mean percent change 30.6 ± 6.5%, $p < 0.05$). The FBF increases in the ID and II genotype groups were significantly different from that in the DD genotype group (Fig. 3).

DISCUSSION

In the present study, we evaluated the relationship between the ACE genotype and the change in the forearm vasodilator response to ERT in postmenopausal women. We found that the change in reactive hyperemia of the forearm peripheral vessels after three months of oral estrogen therapy appeared to be related to the ACE genotype.

Estrogen and endothelial function. Previous studies have shown that the transdermal and oral administration of estrogen improves forearm endothelial function in postmenopausal women (9–12). This is one of the major benefits of estrogen in the prevention of coronary heart disease.

Table 1. Clinical Variables in Each Genotype Group at Baseline and After Three Months of Estrogen Replacement Therapy

Variable	DD (n = 9)		ID (n = 21)		II (n = 25)	
	Baseline	3 Months	Baseline	3 Months	Baseline	3 Months
Body weight (kg)	52.5 ± 2.6	52.7 ± 2.4	54.2 ± 1.8	54.8 ± 1.9	53.5 ± 1.6	54.1 ± 1.9
Body mass index (kg/m^2)	22.1 ± 0.8	22.2 ± 0.6	23.2 ± 0.5	23.5 ± 0.7	22.3 ± 0.6	22.6 ± 0.5
Systolic blood pressure (mm Hg)	132.7 ± 7.7	129.4 ± 7.4	128.0 ± 3.7	124.5 ± 3.1	130.0 ± 4.1	129.3 ± 4.1
Diastolic blood pressure (mm Hg)	82.0 ± 3.7	79.0 ± 4.4	77.8 ± 2.8	75.5 ± 2.6	80.5 ± 4.2	78.6 ± 2.6
Heart rate (beats/min)	62.7 ± 2.2	61.1 ± 1.1	65.9 ± 1.9	66.0 ± 1.9	66.7 ± 2.2	66.9 ± 2.2
Total cholesterol (mg/dl)	237.3 ± 15.7	208.2 ± 12.5*	240.9 ± 8.8	221.1 ± 8.9*	230.0 ± 9.2	213.2 ± 8.0*
Triglycerides (mg/dl)	118.6 ± 109	127.1 ± 7.4	120.1 ± 14.7	126.4 ± 12.2	117.5 ± 22.2	136.6 ± 18.3
HDL cholesterol (mg/dl)	70.7 ± 3.3	75.9 ± 3.7*	72.3 ± 3.3	76.7 ± 3.4*	71.8 ± 3.4	78.8 ± 4.3*
LDL cholesterol (mg/dl)	158.0 ± 19.3	125.1 ± 15.6*	154.7 ± 9.7	136.1 ± 9.8*	145.8 ± 7.6	123.8 ± 7.3*
Estradiol (pg/ml)	12.8 ± 2.1	42.6 ± 2.2*	12.6 ± 1.0	36.5 ± 6.3*	14.5 ± 1.9	46.4 ± 8.3*
Plasma ACE activity (IU/liter at 37°C)	15.0 ± 0.9†	14.4 ± 1.2	13.9 ± 1.0†	12.2 ± 0.9*	11.0 ± 0.4	9.8 ± 0.6*
Nitrite/nitrate (μmol/liter)	26.0 ± 2.0	30.3 ± 3.6	29.3 ± 2.3	39.2 ± 4.1*	28.2 ± 3.7	36.1 ± 4.6*
Basal FBF (ml/min per 100 ml tissue)	6.2 ± 0.5	6.2 ± 0.8	5.8 ± 0.3	5.9 ± 0.4	6.2 ± 0.4	6.1 ± 0.5

*$p < 0.05$ versus baseline (month 0). †$p < 0.05$ versus genotype II group. Results are presented as the mean value ± SE.
ACE = angiotensin-converting enzyme; FBF = forearm blood flow; HDL = high density lipoprotein; LDL = low density lipoprotein.

Estrogen may block the release of endothelium-derived constricting factors (34) or may enhance the release or bio-availability of NO from endothelial cells, resulting in increased cyclic guanosine monophosphate in the underlying smooth muscle and vasorelaxation (35,36). Through its alpha-type receptor, estrogen may directly upregulate eNOS gene expression (14,15). Another possibility is that estrogen-induced changes in lipoprotein levels indirectly result in increased production of NO. For instance, estrogen-induced reductions in LDL and oxidized LDL and increases in HDL should improve endothelial-vascular function overall and diminish vascular disease, thereby increasing vascular NO production (16,17). The mechanism by which estrogen improves endothelial function is thought to be complex and multifactorial.

ACE activity and endothelial function. The renin-angiotensin system has been reported to be suppressed by hormone replacement therapy in hypertensive menopausal women, without an increase in blood pressure, as well as in normotensive postmenopausal women (18,19). Elevated ACE activity might lead to high angiotensin II levels, which might then contribute to increased cardiovascular risk. The ACE inhibitors have been shown, in some clinical studies, to improve endothelial function (20,21). Because ACE is identical to kininase II and is present on the vascular endothelium, bradykinin is inactivated by ACE. Thus, it is assumed that ACE inhibitors may act on kininase II and increase the tissue concentration of bradykinin, which, in turn, augments the vasodilation induced by bradykinin, leading to an increase in NO synthesis (22,23).

Polymorphisms of the ACE gene have been found, and the presence of the deletion (D) allele has been associated with higher concentrations of circulating and tissue ACE (37). The DD genotype has also been associated with an increased risk of coronary restenosis and myocardial infarction in some, but not all, studies. In our present study, the plasma ACE activity correlated with the ACE genotype in Japanese normotensive postmenopausal women. The plasma ACE activity in subjects with the DD genotype was not significantly higher than that in subjects with the ID genotype in our study group, which is consistent with the findings in another hypertensive Japanese group (38).

ACE genotype and vasodilation to estrogen. The purpose of the present study was to determine whether the ACE genotype affects the vasoactive response to ERT in postmenopausal women. Some studies have suggested a relationship between the genotype and the physiologic effects of ACE inhibition, with an attenuation of the beneficial effect noted with the D allele (27,28). In the present study, we found no difference in the baseline FBF responses to reactive hyperemia between the different genotypes, as found in some studies (28,39). However, the increase in the FBF responses to reactive hyperemia with three months of oral estrogen therapy was restricted to subjects with the ID and II genotypes. Although the reason for this observation is not explained by this study, some possibilities might be considered. It is well known that a balance between angiotensin II and NO is important in the regulation of vascular tone (40). Angiotensin II increases vascular superoxide production through the activation of membrane-associated nicotinamide adenine dinucleotide diaphorase/nicotinamide adenine dinucleotide phosphate diaphorase oxidase, resulting in NO inactivation and toxic peroxynitrite production (40). Therefore, ACE inhibitors may increase NO by inhibiting angiotensin II production. Furthermore, under physiologic conditions, endogenous bradykinin is limited by ACE. Bradykinin binds to B_2 receptors on the endothelial cell surface, causing the release of NO (41). The ACE inhibitors prevent bradykinin degradation, leading to increases in NO release. Anderson et al. (28) reported that the percent reduction of ACE activity was greater in those with the II genotype than in those with the DD genotype after the administration of the ACE inhibitor enalapril. In the present study, a significant decrease in plasma ACE activity and an increase in the serum concentration of nitrite/nitrate with ERT were seen in subjects with the ID and II genotypes, but not in those with the DD genotype. This finding suggests that the increase in FBF responses to reactive hyperemia after ERT is related to the degree of inhibition of the plasma ACE concentration. Indeed, we found no significant differences in the changes of other variables, such as HDL cholesterol, LDL cholesterol or body weight, after ERT between the three groups. Furthermore, it may be related to the increased tissue level of ACE, an attenuated interaction with estrogen and the tissue ACE or increased levels of oxidative stress in subjects with the DD genotype. In addition, downregulation of the angiotensin II type receptor in those with the DD genotype has also been recently suggested (42). The duration of the treatment may also be related to the genotype, thus affecting the results (43).

The present study demonstrated that a polymorphism of the ACE gene is a useful predictor of the effect of ERT on forearm reactive hyperemia in postmenopausal women. We believe that this is an instance in which an association between the vasoactive response to ERT in postmenopausal women and ACE genotype has been demonstrated.

Study limitations. A superior method to assess the endothelium may be the direct intra-arterial infusion of a vasoactive agent in the forearm. Recently, Celermajer et al. (44) developed a noninvasive method for producing reactive hyperemia to assess endothelial function. In the present study, we measured the FBF response to reactive hyperemia using strain-gauge plethysmography. This technique is useful for assessing forearm arterial resistance and endothelial function. This technique was simple and reproducible and caused no adverse effects. We recommend this noninvasive technique to assess endothelial function in routine clinical examinations and in future studies.

Several limitations should be considered in assessing this study. First, this was not a double-blind, placebo-controlled

clinical study. Second, subjects were not matched. Also, there were relatively few participants, especially those with the DD genotype, as compared with large epidemiologic trials. However, significant differences in the FBF response to reactive hyperemia after ERT and the plasma concentrations of ACE were observed in the DD genotype group. A more significant difference in these variables might be found in a similar study with a larger sample size.

Conclusions. The present study suggests that the effect of ERT on forearm reactive hyperemia may be determined in part by the ACE genotype in postmenopausal women.

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