Time Since Menopause Influences the Acute and Chronic Effect of Estrogens on Endothelial Function

Cristiana Vitale, Giuseppe Mercuro, Elena Cerquetani, Giuseppe Marazzi, Roberto Patrizi, Francesco Pelliccia, Maurizio Volterrani, Massimo Fini, Peter Collins, Giuseppe M.C. Rosano

Objective—We evaluated whether time since menopause influences the acute and chronic effect of Estradiol (E) on vascular endothelial function.

Methods and Results—We studied flow-mediated dilatation (FMD) in 134 postmenopausal women (PMW) before and after acute and chronic E administration. At baseline FMD was inversely associated to time from menopause ($r = -0.67$, $P < 0.001$) and age ($r = -0.43$, $P < 0.05$), in exogenous estrogen naïve but not in previous users. Acute and chronic E improved endothelial function in all women. E administration improved FMD more in women within 5 years since menopause than in those with more than 5 years since menopause (76% and 74% versus 45% and 48%, acute and chronic E, respectively; $P < 0.05$). Among women with more than 5 years since menopause acute and chronic E increased FMD more in previous E users than in nonusers (59% and 63% versus 31% and 38%, acute and chronic E, respectively; $P < 0.01$). Multivariate analysis showed that time from menopause was a predictor of impaired FMD and of its improvement after acute and chronic E.

Conclusions—Time from menopause influences FMD in PMW. The acute and chronic effect of E on FMD is time dependent and is reduced by a longer time since menopause. (Arterioscler Thromb Vasc Biol. 2008;28:348-352)

Key Words: endothelium • endothelial function • cardiovascular disease prevention • risk factors • estrogen

Endothelial dysfunction is an initial step in the development of atherosclerosis and is associated with the progression of atherosclerosis and with cardiovascular events in men and women. Although aging is associated independently with a progressive decline in endothelium-dependent vasodilatation in both sexes, age-related endothelial dysfunction is attenuated in premenopausal women, compared with men of similar age. However, no difference in endothelial function between sexes is observed after the 5th decade, suggesting a protective effect of endogenous ovarian hormones on endothelial function in women.

Several studies have shown that estrogen or estrogen-progestin replacement therapy (ERT or HRT) restore, at least in part, the impairment of endothelial function related to the cessation of menses. In spite of a large body of evidence suggesting favorable effects of estrogens alone or in combination with progestins on surrogate markers of cardiovascular disease and on cardiovascular events, recent randomized controlled trials (RCT) failed to show a significant reduction in cardiovascular events with ERT or HRT in predominantly late postmenopausal women (PMW). It has been suggested that the discrepancy between observational and RCT may be dependent on the timing of the initiation of ERT or HRT and on the different cardiovascular effects of ovarian hormones in younger and older women. Recent studies have suggested that the vascular effect of ERT or HRT is age dependent; however, it is not known whether the vascular effect of estrogens is related purely to age or more to the duration of estrogen deficiency and whether previous exposure to ERT may influence the vasodilator effect of estrogens in women.

The aim of the present study was to evaluate the effect of time since menopause and previous hormone use on the acute and chronic effect of Estradiol (E) on endothelial function in PMW.

Methods

Study Population

The study population included 162 consecutive PMW aged between 46 to 72 years, referred from the local menopause clinic for a routine cardiovascular assessment before the start of ERT or HRT, over an 18-month period. A full medical history and physical examinations were performed to evaluate the degree of cardiovascular risk, and each woman was asked to provide detailed information about her past use of ovarian hormone replacement therapy. All women underwent full cardiac evaluation, including echocardiogram and exercise testing if necessary.

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Women were included if they had been amenorrheic for at least 6 months and had follicle-stimulating hormone (FSH) levels $>40$ mIU/mL and 17$\beta$-estradiol levels $<30$ pg/mL. Only women with no contraindications for ERT or HRT, no evidence or suspicion of cancer, and no history of venous thromboembolism were included in the study. Before participation, all women gave written informed consent to the study which had been previously approved by the Ethics Committee of the San Raffaele EUR Hospital, Rome. Women with ischemic heart disease, primary valvular disease, or myocardial disease were excluded as those with contraindications to ERT or HRT. Women were excluded from participating if they had used ERT or HRT or selective estrogen receptor modulators (SERMs) and had discontinued within the last 6 months or had an ovariectomy in the past 3 months.

Study Design
After a baseline evaluation, women underwent study of endothelial function by means of FMD of the brachial artery and blood sampling. The assessment of endothelial function was repeated within 1 hour ($\geq 40-60$ minutes) after acute administration of E (1 mg s.l.) and after 3 months of oral ERT (estradiol valerate 1 mg/d). Blood sampling was performed through a venous line, positioned in the contralateral arm to that on which endothelial function was studied, 15 minutes before the endothelial function study and was kept open by continuous saline infusion throughout the study. Blood sampling was performed at baseline and at the end of each endothelial function study. E2 plasma levels were assessed with a Micro-particle Enzyme Immunoassay (MEIA) on the AxSYM System (Abbott Laboratories).

Brachial Artery Endothelial Function
Endothelial function was assessed with high-resolution ultrasound by measuring changes from baseline in the caliber of the brachial artery during reactive hyperemia. Studies of endothelial function and image analysis were conducted according to a previously described protocol$^{18}$ (please see supplemental materials, available online at http://atvb.ahajournals.org).

Statistical Analysis
An explanatory analysis was performed and means values with corresponding standard deviation values were provided. Counts were expressed in percentages where appropriate. Because of the skewed distribution of endothelial function a nonparametric test (Wilcoxon signed-rank test) was used to compare median values at baseline and at follow-up assessment. Correlation between variables was calculated with Spearman correlation coefficient. Stepwise multivariate logistic regression analysis was performed to investigate independent determinants of improvement in endothelial function using age, time since menopause, baseline E levels, previous use of oral contraceptives, total cholesterol levels, cigarette smoking, body mass index (BMI), diabetes mellitus, arterial hypertension, and previous hormone use (in estrogen users) as covariates. All variables were selected a priori. A univariate analysis was performed to detect variables associated with the endothelial response. Variables which showed a significant association with endothelial function were entered into a Cox Proportional Hazard Model multivariate analysis. A probability value $<0.05$ was accepted as the level of statistical significance. All tests were performed with a commercially available GBStat software package.

Results
From a group of 162 consecutive women referred, 134 women, aging from 46 to 72 years (mean age 62 $\pm$ 6 years), matching study entry criteria were selected and entered in the screening phase of the study. Twenty-eight women were not included in the study because either unwilling to take ERT (15 patients) or because of contraindications to ERT (8 patients) or because they did not meet the inclusion criteria (5 patients). A total of 6 women did not complete the study. Of the 6 women that did not complete the study protocol 1 was lost at follow-up because of a move to another city, 4 because advised so by the general practitioner, and 1 because of breast tenderness. The baseline clinical characteristics of the study participants are given in Table 1. At entry Follicle Stimulating Hormone was 68.2 $\pm$ 11.2 IU/L and plasma 17$\beta$-estradiol levels $<30$ pg/mL in all women. Eighteen patients had arterial hypertension and 26 patients were hypercholesterolemic; all hypertensive and hypercholesterolemic women were treated to achieve therapeutic goals (SBP $\leq 140$ mm Hg, DBP $\leq 90$ mm Hg, total cholesterol $<200$ mg/dL, LDL cholesterol $<130$ mg/dL, triglycerides $<150$ mg/dL). Nine patients had type 2 diabetes mellitus treated with either diet alone or in association with oral antidiabetic agents, and all had HbA1c levels $<7%$. All patients were taking drugs known to affect endothelial function for 6 months or more before the start of the study and no significant changes to the dose or to the type of cardiovascular drugs were made during the study period. No patients were taking soy or herbal supplements. Among the 134 PMW included in the study, 35 had never used ERT or HRT (exogenous estrogen naive) whereas 18 had used either diet alone or in association with oral antidiabetic agents, and all had HbA1c levels $<7%$. All patients were taking drugs known to affect endothelial function for 6 months or more before the start of the study and no significant changes to the dose or to the type of cardiovascular drugs were made during the study period. No patients were taking soy or herbal supplements. Among the 134 PMW included in the study, 35 had never used ERT or HRT (exogenous estrogen naive) whereas 18 had used either ERT or HRT for less than 3 months in the past and had discontinued the therapy by more than 24 months and those were categorized as nonusers, 81 patients had been using either ERT or HRT for at least 6 months before their inclusion in the study. Past hormone users were older and had a longer time since menopause (Table 1).

Table 1. Baseline Clinical Characteristics of Study Participants (n=134)

<table>
<thead>
<tr>
<th></th>
<th>All (n=134)</th>
<th>Exogenous estrogen naive (n=53)</th>
<th>Past users (n=81)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62$\pm$6</td>
<td>56.5$\pm$4.5</td>
<td>66$\pm$4*</td>
</tr>
<tr>
<td>Years since menopause</td>
<td>13.8$\pm$2.5</td>
<td>5$\pm$2</td>
<td>16$\pm$4*</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>26.2$\pm$1.7</td>
<td>25.6$\pm$1.4</td>
<td>27.1$\pm$22</td>
</tr>
<tr>
<td>Cigarette smokers</td>
<td>12</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Hypertension</td>
<td>18</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>26</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Diabetics</td>
<td>9</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Baseline clinical features of study subjects. Exogenous estrogen naive were older and had a longer time since menopause. *P<0.05.

Baseline Endothelial Function Study
Overall baseline FMD was 5.6$\pm$1.5%, FMD was reduced in women aged $\geq 60$ years compared with those aged between 50 to 59 years (4.9$\pm$1.3% versus 6.6$\pm$0.9%, $P<0.05$). FMD was inversely associated to time since menopause ($r=-0.67$, $P<0.001$) and age ($r=-0.43$, $P<0.05$) in nonhormone users whereas no correlation was found in prior hormone users. No significant correlation between FMD and either time since menopause or age was found when nonhormone users were pooled with previous hormone users.

Effect of Acute and Chronic Estradiol Administration
Both sublingual and chronic oral E administration increased estradiol plasma levels in all women. The estradiol plasma levels rose from 23.42$\pm$9.41 pg/mL to 624.82$\pm$148.65

Statistical Analysis
An explanatory analysis was performed and means values with corresponding standard deviation values were provided. Counts were expressed in percentages where appropriate. Because of the skewed distribution of endothelial function a nonparametric test (Wilcoxon signed-rank test) was used to compare median values at baseline and at follow-up assessment. Correlation between variables was calculated with Spearman correlation coefficient. Stepwise multivariate logistic regression analysis was performed to investigate independent determinants of improvement in endothelial function using age, time since menopause, baseline E levels, previous use of oral contraceptives, total cholesterol levels, cigarette smoking, body mass index (BMI), diabetes mellitus, arterial hypertension, and previous hormone use (in estrogen users) as covariates. All variables were selected a priori. A univariate analysis was performed to detect variables associated with the endothelial response. Variables which showed a significant association with endothelial function were entered into a Cox Proportional Hazard Model multivariate analysis. A probability value $<0.05$ was accepted as the level of statistical significance. All tests were performed with a commercially available GBStat software package.
Table 2. Effect of Acute and Chronic Estrogen Administration on Endothelial Function

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After Acute Estradiol</th>
<th>After Chronic Estradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial artery diameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal-1, mm</td>
<td>4.11±0.33</td>
<td>4.11±0.29</td>
<td>4.12±0.29</td>
</tr>
<tr>
<td>Hyperemia, mm</td>
<td>4.34±0.63</td>
<td>4.49±0.62‡</td>
<td>4.51±0.78†</td>
</tr>
<tr>
<td>FMD, %</td>
<td>5.6±1.5</td>
<td>9.3±0.6‡</td>
<td>9.6±1.3†</td>
</tr>
<tr>
<td>Basal-2, mm</td>
<td>4.11±0.38</td>
<td>4.11±0.69</td>
<td>4.12±0.72</td>
</tr>
<tr>
<td>Nitroglycerine, mm</td>
<td>4.83±0.62</td>
<td>4.82±0.59</td>
<td>4.83±0.56</td>
</tr>
<tr>
<td>Estradiol 17β, pg/ml</td>
<td>23.42±9.41</td>
<td>624.82±148.65</td>
<td>107.4±44.86</td>
</tr>
</tbody>
</table>

Acute and chronic effect of estradiol 17β on endothelial function of PMW. FMD, flow mediated dilation. Data is expressed as means±1 SD. †P<0.01.

pg/mL after acute E administration and to 107.4±44.86 pg/mL after chronic estradiol administration. Analysis of baseline brachial artery diameter before reactive hyperemia revealed no significant treatment effects on resting vessel diameter (Table 2). FMD was increased by both acute and chronic E administration in all women (Table 2). Compared with baseline, acute E administration improved FMD by 64% whereas chronic E therapy improved FMD by 72%. No significant difference in the degree of improvement of FMD after acute and chronic E was detected between nonusers and past users. Among exogenous estrogen naïve E administration improved FMD more in those women within 5 years since menopause than in those with more than 5 years since menopause (Figure 1). Among women with more than 5 years since menopause acute and chronic E increased FMD more in those who have received hormones in the past than exogenous estrogen naïve women (Figure 2).

Endothelium-independent vasodilatation after sublingual nitroglycerin was not affected by the acute and chronic E administration.

An inverse correlation was found between improvement in FMD after both acute and chronic E and time since menopause exogenous estrogen naïve women (r=0.56 and 0.61 respectively; P<0.05) but not in past hormone users. When prior hormone users were pooled to exogenous estrogen naïve the correlation was lost (r=0.23, P=0.52).

In exogenous estrogen naïve, univariate analysis showed that both time from menopause and age were predictors of impaired endothelial function and its improvement after estrogen therapy. In the multivariate analysis only time since menopause was found as an independent variable associated with both impaired endothelial function and its improvement with estrogen.

In all women, univariate analysis showed that time since menopause (P<0.0002), age (P<0.001), baseline estradiol levels (P<0.001), cigarette smoking (P<0.002), past use of oral contraceptives (P<0.002) and BMI (P<0.002) were variables associated with impaired estrogen-induced improvement of endothelial function. In exogenous estrogen naïve, univariate analysis showed that both time since menopause (P<0.0005) and age (P<0.002) were predictors of endothelial function improvement after estrogen therapy.

From the multivariate analysis, time since menopause was found to be the only variable that at the 5% level of statistical significance was independently associated with impaired endothelial function and its improvement with estrogen both in all women and in estrogen naïve women (2.8 [3.6 to 1.4] P=0.003, 3.5 [5.1 to 1.7] P<0.0001), respectively. Time since menopause and endothelial function improvement were negatively correlated, suggesting that as time since menopause increases, endothelial function and its improvement with estrogens are reduced.

Discussion

The present study shows that in PMW time since menopause influences endothelial function and that the effect of E on FMD is related to the time elapsed since menopause. Indeed we found that endothelial function correlates inversely with age and time since menopause in exogenous estrogen naïve PMW whereas past hormone use reduces the detrimental

Figure 1. Improvement in FMD after acute and chronic estradiol administration in exogenous estrogen naïve. FMD improved significantly more (P<0.05) in women <5 years since menopause than in women >5 years since menopause.

Figure 2. Improvement in FMD after acute and chronic estradiol administration in women ≥5 years since menopause. FMD improved significantly more (P<0.01) in those women who had received estrogens in the past than in those who exogenous estrogen naïve.
effect of aging on vascular reactivity and improves endothelial response to E administration. This suggests that postmenopausal ovarian hormone use buffers the detrimental effect of estrogen deprivation on endothelial function.

Our results also suggest that the time of initiation of ERT or HRT more than aging is the key factor in determining the vascular responsiveness to estrogen in PMW. The recent reanalysis of the WHI results has underlined the importance of timing of initiation of HRT, showing that HRT is associated with fewer cardiovascular events and total mortality (30% of reduction) if initiated in younger women (50 to 59 years old) or within 10 years after menopause, whereas it has no protective effect in older women (> 60 years old) or in those with more than 10 years after menopause.\textsuperscript{19} More recently the results of the WHI Coronary-Artery Calcium Study (WHI-CACS), an ancillary study evaluating the effects of estrogen on coronary-artery calcification in women in the WHI-CEE trial who were 50 to 59 years old at randomization,\textsuperscript{20} have shown that women who received estrogens had significantly less coronary-artery calcification than women receiving placebo. All these data suggest that there is a time-window for the cardiovascular effect of estrogens in PMW and may help to better understand the apparently divergent cardiovascular findings of observational and randomized studies with ERT or HRT.\textsuperscript{21–23} Indeed, in the observational studies women started ERT or HRT mainly for menopausal complaints and within a few years since menopause, whereas in the randomized studies ERT or HRT was started predominantly in asymptomatic women and mainly aging >60 years.\textsuperscript{24–26}

Our results, showing that the vascular effect of ERT is time-dependent and is reduced by longer time lapsed from the menopause, suggest that the physiological effect and the balance between favorable and unfavorable vascular effects of exogenous estrogens, differ according to time since menopause suggesting a window of opportunity for HRT and suggesting a biological plausibility for a favorable effect of estrogens in early PMW. The findings of this study are in agreement with the proposed “timing hypothesis” for HRT, which suggests that the benefits of HRT in preventing atherosclerosis occur only when the therapy is initiated before advanced atherosclerosis develops, as atherosclerosis in women tend to develop in a time-dependent manner after the menopause.\textsuperscript{21,26,27}

These findings are likely to be dependent on the effect of estrogen deprivation on estrogen receptor expression and functioning.\textsuperscript{28} Recent findings suggest that the decreased vascular effects of estrogens may be dependent not only on the plasma levels of estrogens but also on the possible age-related changes in the number of estrogens receptors (Ers) or the signaling mechanisms downstream from Ers stimulation. Post et al have shown that methylation of the promoter region of the ER alpha gene in the vascular system is age-dependent and results in an inactivation of gene transcription.\textsuperscript{29} Therefore, a longer time since menopause and a longer exogenous estrogen exposure may be crucial in regulating ER response to exogenous estrogen administration. Results from this study show that the responsiveness of the endothelium to E is preserved in women who have received ERT or HRT in the past suggesting that postmenopausal supplementation of estrogen may reduce the inactivation of vascular ERs.

To this end estrogens will exert a favorable effect on endothelial function and on arterial health if ERT or HRT is initiated soon after menopause when the ERs are still present and functioning. On the other hand if ERT or HRT is started late after the menopause most of the beneficial cardiovascular effect will be lost and the prevailing procoagulant effect of estrogens may increase the incidence of vascular events.\textsuperscript{30–31}

Conversely to a recent report of Sherwood et al that suggested that the vascular effect of ERT or HRT is dependent on age\textsuperscript{17} we found that time since menopause and not age influences the acute and chronic vascular response to E. Furthermore, Sherwood et al reported that previous hormone use was not a significant determinant of FMD response to transdermal ERT or HRT. This disagreement may be related to the different route of administration of estrogens and patient populations under study as our women were all healthy and younger than those reported in the study of Sherwood et al where more than 80% of women had more than 60 years.

They have also shown that the adjunct of progestin (NETA) to estrogens abolishes the beneficial effect of E on endothelial function.\textsuperscript{18} Progestins have vascular actions which depend on their chemical structure, dose, and route of administration and that these factors may attenuate the beneficial effect of estrogens on endothelial function.\textsuperscript{32} Furthermore, several studies have shown different effects of different progestins on endothelial function and thrombotic risk.\textsuperscript{32–35} The WHI study has shown that in nonhysterectomized women receiving CEE-MPA in continuous combined scheme increased cardiovascular events in elderly PMW, whereas this was not the case in women receiving CEE alone.\textsuperscript{10,11}

Limitations of the present study are the lack of placebo control and of the adjunctive use of progestins. The inclusion of a placebo arm could have been useful to further confirm the well known effect of estrogens on endothelial function. However, because the main aim of the present study was to assess the physiological effects of estrogens related to time since menopause we chose not to have a placebo arm nor to include progestins as a variable. Therefore, the lack of progestin use does not allow to translate the results of our study to estrogen-progestin therapy.

Also, although as suggested by the ESTHER study,\textsuperscript{31} transdermal administration of E may reduce the incidence of venous thromboembolism and may have beneficial vascular effects, we have chosen to use the oral route because previous studies found a greater and a more reliable effect of oral administration than transdermal administration on endothelial function.\textsuperscript{36–37}

Some of our patients were receiving therapy for the control of cardiovascular risk factors, known to affect endothelial function. However, since they had been using the cardiovascular medications for a period of at least 6 months before study and no significant change in dose or type of medications was adopted during the study period, it is unlikely that these factors may attenuate the beneficial effect of E on endothelial function and cardiovascular outcomes.
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Disclosures
None.

References
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Material and Methods

Brachial Artery Endothelial Function

All patients were studied fasting in a quiet, temperature-controlled room (22° to 23°C). Baseline and follow-up studies of endothelial function were conducted at the same hour of the day ± 1 hour in each patient. Participants were asked to avoid drinking beverages containing caffeine and to refrain from smoking for 12 hours preceding the study. Following an initial 15-minute rest period in which patients were placed in a supine position, the brachial artery of the dominant arm was imaged using a Sonos 2500 high-resolution ultrasound machine (Hewlett Packard Sonos 2005) equipped with a 7.5 to 12.5 MHz linear-array transducer. The artery was scanned over a longitudinal section 3 to 5 cm above the elbow. The diameter of the right brachial artery was measured at rest, during reactive hyperemia, after a 10-minute recovery period, and 5 minutes following sublingual nitroglycerin. A pneumatic tourniquet was placed around the forearm proximal to the target artery and was inflated to a pressure of 50 mmHg above patients systolic blood pressure for 5 minutes. Reactive hyperaemia was induced by sudden cuff deflation. To assess endothelium-independent vasodilatation, sublingual nitroglycerin (0.4 mg) was administered, and a fourth scan was recorded for 5 minutes.

Image Analysis. Endothelial function studies were performed in each patient by the same investigator who was unaware of the study data. Image analysis was performed using a validated program. The diameter of the brachial artery was measured from the anterior to the posterior interface. The mean arterial diameter was calculated from 4 cardiac cycles synchronized with the R-wave peak on the electrocardiogram. All measurements were made at end diastole. The diameter change was expressed as the percent change compared with baseline diameter. In our group the
intra-observer variability in diameter measurements is 0.38% ± 0.26% (range, 0.1%–1.2%),
yielding a coefficient of variation of 1.26% and a coefficient of repeatability of 0.5%.