Effects of short-term transdermal estradiol administration on plasma levels of nitric oxide in postmenopausal women

Ettore Cicinelli, M.D.,* Louis J. Ignarro, M.D.,† Luca Maria Schönauer, M.D.,* Maria Giuseppina Matteo, M.D.,* Pietro Galantino, M.D.,* Gabriella Balzano, M.D.*

University of Bari, Bari, Italy, and University of California, Los Angeles, Los Angeles, California

Objective: To assess the effects of short-term transdermal E2 administration on nitric oxide (NO) plasma levels in postmenopausal women.

Design: Randomized, placebo-controlled trial.

Setting: Healthy volunteers in an academic research environment.

Patient(s): Twenty-eight healthy postmenopausal women.

Intervention(s): Transdermal administration of E2 (100 µg/d) or placebo on days 1 and 4 of a 1-week treatment regimen.

Main Outcome Measure(s): Serum concentrations of E2 and plasma concentrations of NO stable oxidation products were assessed on day 1, before placement of the patch, and subsequently on days 2, 3, and 6.

Result(s): The mean concentration of NO metabolites on days 2, 3, and 6 was significantly greater in the E2 group (40.08 ± 15.42 pmol/L, 38.05 ± 18.82 pmol/L, and 42.03 ± 16.81 pmol/L on days 2, 3, and 6, respectively) compared with both baseline levels (23.07 ± 5.79 pmol/L) and the placebo group (23.51 ± 4.06 µmol/L, 21.64 ± 4.72 µmol/L, and 21.81 ± 4.46 µmol/L on days 2, 3, and 6, respectively).

Conclusion(s): During a 1-week treatment regimen with transdermal E2, plasma levels of NO in postmenopausal women were significantly higher than baseline levels on days 2, 3, and 6. This suggests that the effect of estrogens on NO synthesis is rapid and that it is maintained with repeated administration. (Fertil Steril 1998;69:58-61. ©1998 by American Society for Reproductive Medicine.)

Key Words: Estradiol, transdermal administration, nitric oxide, postmenopausal women, hormone replacement therapy, nitrite

It currently is estimated that the favorable changes in serum lipids and lipoproteins that are induced by postmenopausal estrogen replacement therapy (ERT) may account for only 30% of the reduction in the incidence of cardiovascular disease that is observed in the treated population (1). Additional mechanisms, such as the direct effect of estrogens on arteries, may play an important role in cardioprotection.

Estrogens induce vasodilation in both pelvic and extrapelvic arteries (2-4), and they potentiate the endothelium-dependent vasodilation in both conducance and resistance arterial districts (5, 6). Several studies support the hypothesis that estrogens influence the release of nitric oxide (NO) from the vascular endothelium (7-11). It is known that NO mediates the vascular relaxation produced by various endothelium-dependent vasodilators, such as acetylcholine and bradykinin (12, 13).

Rosselli and co-workers (11) observed a significant increase in NO plasma levels in postmenopausal women who were treated for...
at least 6 months with continuous administration of E2 (50 μg/d) plus norethisterone acetate (1 mg/d) on days 1 through 12 of each month. These investigators speculated that the effects of estrogens were rapid and receptor-operated, and that the upregulation of estrogen receptors in the vasculature of postmenopausal women contributed to the increased NO synthesis.

Accordingly, we recently have demonstrated that plasma levels of NO in postmenopausal women 24 hours after transdermal E2 administration are significantly higher than baseline levels, and that the percentage increase in NO plasma levels correlates with serum levels of E2 (9). For a better understanding of mechanisms involved in the estrogen-induced increase in NO production, we measured NO plasma levels on different days during short-term (1-week) E2 treatment in postmenopausal women.

The production of NO, which is an extremely labile molecule, was assessed by monitoring plasma levels of nitrite and nitrate, the two stable oxidation products of NO metabolism. Levels of these stable compounds have been used as markers for NO synthase activity in vivo (8-11, 14). Metabolic tracer studies in humans with the use of L-[guanidino-15N]arginine have demonstrated that increased nitrate production in serum in vivo was derived from NO generated from the terminal guanidino nitrogen atom of labeled L-arginine (15).

MATERIALS AND METHODS

The study, designed as a randomized, placebo-controlled trial, was approved by the institutional review board at our institution. Twenty-eight women in spontaneous postmenopause who had not undergone hysterectomy were enrolled in the study. All subjects were counseled about the nature and purpose of the study and signed a detailed consent form.

Menopausal status was confirmed by the absence of menstruation for at least 1 year and a serum concentration of FSH of >40 mIU/mL. No women had been in menopause for longer than 10 years, and they all were of normal weight. None had ever received hormone replacement therapy or had had coronary artery disease, hypertension, or diabetes, and none had taken any medication known to affect blood pressure within the previous 12 months. Alcohol, caffeine, and smoking were prohibited for 24 hours before and during the study.

Women were assigned randomly to receive transdermal E2 patches (100 μg/d, Estraderm 0.1; CIBA Pharmaceutical, Basel, Switzerland) or a nonmedicated patch on days 1 and 4 of the 1-week treatment period. Randomization was done by opening sealed envelopes containing computer-generated block randomization numbers. Blood samples were collected at 8 a.m. after overnight fasting on day 1, before placement of the patch, and on days 2, 3, and 6. Women in both groups were given the same meals containing aliments with poor nitrite content beginning 24 hours before the first blood sampling and continuing throughout the study period.

All blood samples were divided into citrated and noncitrated tubes and centrifuged immediately in a refrigerated centrifuge at 2,000 × g for 10 minutes; both the plasma and the serum were frozen at -20°C until assayed. Serum E2 (normal menopausal values, <20 pg/mL) was assayed with the use of a no-extraction iodine-125 RIA (DRI RIA-ESTR; Sorin Biomedica, Saluggia, Italy). The sensitivity of the assay was 10 pg/mL and the variation coefficient was <6% in the low range of values.

Nitric oxide production was assessed by monitoring plasma levels of nitrite and nitrate, the two stable oxidation products of NO metabolism, using procedures that were described previously by this laboratory (14). Briefly, chemiluminescence detection was used to quantify both nitrite and nitrate in samples of plasma that were deproteinized by mixing two volumes of cold ethanol with one volume of plasma, then centrifuging to remove the precipitate. Supernatant fractions (0.1 mL) were analyzed by refluxing in acidic vanadium (III) under argon as described previously (14).

This method detects and is quantitative for nitrite plus nitrate, both of which are reduced to NO, which subsequently is assayed by chemiluminescence detection. This technique is more sensitive than standard colorimetric methods, and it has been validated and used to monitor NO formation both in vitro and in vivo (14).

Clinical characteristics of the women in both groups were compared by unpaired Student's t-test. The variation in NO metabolites and E2 concentrations was evaluated statistically with the use of analysis of variance and Student-Newman-Keuls test. Concentrations in the two groups were compared by unpaired Student's t-test; 95% confidence intervals also were calculated for the observed mean differences. All values were given as means ± SD. P < 0.05 was defined as statistically significant.

RESULTS

The E2 and placebo groups were shown to be homogeneous for age (54.42 ± 2.37 years versus 55.35 ± 2.68 years), years since menopause (4.07 ± 2.61 years versus 5.07 ± 1.94 years), and body mass index (23.25 ± 1.33 kg/m² versus 22.99 ± 1.54 kg/m²). The concentrations of NO stable metabolites and E2 on different days are reported in Table 1.

Baseline E2 serum concentrations were in the menopausal range in both groups. In women who received medicated patches, serum E2 concentrations on days 2, 3, and 6 were significantly higher than baseline concentrations (F = 332.16, df = 52, P < 0.000001) and higher than the corresponding concentrations in the control group.
TABLE 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment day</th>
<th>E₂ group (mean ± SD)</th>
<th>Placebo group (mean ± SD)</th>
<th>Mean difference (95% CI)</th>
<th>Unpaired Student’s t-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₂ (pg/mL)</td>
<td>1</td>
<td>13.78 ± 3.66</td>
<td>15.00 ± 3.39</td>
<td>1.21 (-1.52-3.94)</td>
<td>0.91</td>
<td>0.371</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>103.71 ± 11.87</td>
<td>12.14 ± 2.62</td>
<td>91.57 (84.89-98.25)</td>
<td>28.19</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>83.28 ± 7.96</td>
<td>13.21 ± 5.94</td>
<td>69.07 (62.20-74.94)</td>
<td>29.52</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>79.28 ± 6.22</td>
<td>15.43 ± 2.17</td>
<td>63.86 (60.24-67.48)</td>
<td>36.27</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>NO₂/NO₃ (umol/L)</td>
<td>1</td>
<td>27.44 ± 5.45</td>
<td>27.73 ± 3.49</td>
<td>0.31 (-3.25-3.87)</td>
<td>0.18</td>
<td>0.860</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>40.08 ± 15.42</td>
<td>23.51 ± 4.06</td>
<td>16.58 (7.82-25.34)</td>
<td>3.89</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>38.05 ± 18.82</td>
<td>21.64 ± 4.72</td>
<td>16.41 (5.74-27.08)</td>
<td>3.16</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>42.03 ± 16.81</td>
<td>21.81 ± 4.46</td>
<td>20.22 (10.66-29.78)</td>
<td>4.35</td>
<td>&lt;0.0002</td>
</tr>
</tbody>
</table>

Mean baseline concentrations of NO metabolites were similar in both the treated and placebo groups. At subsequent evaluations, they increased significantly only in the E₂ group (F = 4.97, df = 52, P < 0.005), in which mean levels of NO metabolites on days 2, 3, and 6 were significantly higher than baseline levels. On the same days, the levels of NO metabolites in the E₂ group also were significantly higher than those in the control group.

**DISCUSSION**

Short-term ERT improves flow-mediated endothelium-dependent vasodilation in postmenopausal women (6). The data in this study demonstrate that this vasodilatory effect may be related to a significant increase in plasma levels of NO induced by estrogens, and they provide further evidence that an NO-mediated mechanism may contribute to the cardioprotective effects of ERT.

The effect of estrogens on NO metabolism already has been investigated in vitro and in vivo, in both animals and humans (7, 8, 10). However, few studies have evaluated the effect of ERT on NO metabolism in postmenopausal women. Rosselli and coworkers (11) reported long-term data of postmenopausal women treated with E₂ combined with norethisterone acetate. In a previous study, we demonstrated that transdermal administration of E₂ in postmenopausal women induces a significant increase in NO plasma levels 24 hours after administration (9). Therefore, we undertook this study to evaluate whether the beneficial effect of E₂ on NO metabolism is maintained with repeated administration.

According to our previous findings, plasma levels of NO metabolites 24 hours after E₂ administration (day 2 of treatment) were significantly higher than baseline levels (9); at subsequent evaluations, on days 3 and 6, the levels did not vary significantly from those observed on day 2. This suggests two things: [1] that the positive effect of E₂ on NO metabolism does not decrease with subsequent administration, and [2] that the effect of estrogens is rapid, probably reaching its peak within the first 24 hours of therapy. The results of this study also indicate that the effect of estrogens does not increase with subsequent administration, which is consistent with the finding that changes in steroid nuclear receptors in most circumstances occur within hours (16).

Our results agree in part with those of Rosselli and co-workers (11), who also reported low baseline values of NO metabolites in postmenopausal women, probably in relation to a postmenopausal hypoestrogenic situation, although NO plasma levels during therapy were about 35% lower. We can only speculate on whether this difference is due to the higher E₂ dose used in our study or to the effects of norethisterone acetate.

Nitric oxide is a labile molecule with a half-life of a few seconds that degrades to the stable metabolites, nitrite and nitrate (7–11, 14). Plasma levels of nitrite are known to be extremely low (approaching zero) in most normal individuals, but plasma levels of nitrate are reasonably constant at around 25 to 45 μmol/L (17).

The baseline concentrations of NO metabolites in both study groups were slightly lower than 25 μmol/L, probably as a result of the postmenopausal E₂ deficiency. At subsequent evaluations, the concentrations of NO metabolites increased significantly only in women who were treated with transdermal E₂.

This agrees with the previous findings of Ramsay and associates (10) in premenopausal women who were pretreated first with GnRH analogues, then with oral estrogens. Our results also demonstrate that estrogen-induced NO production occurs in postmenopausal women, who are much older than those studied by Ramsay and associates (10). Because our study was drug- and diet-controlled, only differences in E₂ absorption and NO metabolism could account for the wide intersubject variability that we observed on days 2, 3, and 6 in the percentage increase in the concentrations of NO metabolites.

We can only guess which cells are the main source of the
observed increase in circulating concentrations of NO metabolites because several cell types other than endothelial cells also are known to produce NO (18). Further, only endothelial cells and neuronal cells have both constitutive and inducible forms of NO synthase, whereas most of the other cell types have only inducible NO synthase (18). Because \( E_2 \) stimulates the basal release of NO from endothelium (7), it is possible that the increases in circulating levels of nitrite and nitrate that we observed are solely endothelium-derived.

In conclusion, short-term transdermal administration of \( E_2 \) to healthy postmenopausal women induces a rapid and sustained increase in plasma concentrations of NO compared with baseline levels. Plasma levels of NO on day 2 of treatment were similar to those on day 6 of treatment. This finding supports the hypothesis that the effect of estrogens on NO synthesis is rapid, probably receptor-operated, and maintained with repeated administration. This study provides further evidence that an NO-related mechanism may contribute to the cardiovascular protective effect of ERT in postmenopausal women.

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References