Chronic 17β-Estradiol Replacement Increases Nitric Oxide–Mediated Vasodilation of Guinea Pig Coronary Microcirculation

Loren P. Thompson, PhD; Gerard Pinkas, BS; Carl P. Weiner, MD

Background—Estrogen is cardioprotective of the coronary circulation by mechanisms incompletely understood. This study determined the effect of chronic 17β-estradiol replacement on dilator responses to acetylcholine and sodium nitroprusside of the isolated coronary microcirculation.

Methods and Results—Adult female guinea pigs were ovariectomized, and a 21-day-release pellet containing 0.0, 0.1, 0.25, 0.5, or 1.0 mg 17β-estradiol was implanted subcutaneously. Serum estradiol concentrations ranged from 3.9 to 74.9 pg/mL, increasing with the dose of estradiol. After 19 to 20 days, the animals were euthanized, and their hearts were removed and perfused with buffer at constant flow on an isolated heart apparatus. Both perfusion pressure and contractile force were measured in prostaglandin F2α–constricted hearts. Vasodilation to the cumulative addition of the endothelium-dependent agonist acetylcholine (10−7 to 10−5 mol/L) and the nitric oxide (NO) donor sodium nitroprusside (10−9 to 10−5 mol/L) was measured before and after NO synthesis inhibition by nitro-L-arginine (LNA, 10−4 mol/L). Baseline coronary resistance was unaltered by estradiol, although LNA increased resistance in estradiol-treated hearts more than in ovariectomized controls. Chronic 17β-estradiol increased sensitivity (measured by −log EC50 values) but not maximal response to acetylcholine compared with ovariectomized controls. Differences were abolished by LNA at all doses of estradiol. Sodium nitroprusside–induced dilation was unaffected by estradiol replacement.

Conclusions—Chronic 17β-estradiol replacement, at doses producing hormone levels within the physiological range, enhances dilator sensitivity of the coronary microcirculation through enhanced NO production by the endothelium, independent of changes in NO sensitivity of the vascular smooth muscle. Thus, estradiol enhances NO production as a protective mechanism of the coronary microcirculation. (Circulation. 2000;102:445-451.)

Key Words: acetylcholine ■ hormones ■ nitric oxide ■ vasodilation ■ endothelium

The incidence of coronary artery disease is lower in premenopausal women than in age-matched men.1,2 After the menopause, the risk of coronary artery disease rises to levels equivalent to those of men.1–4 Epidemiological and clinical evidence suggests that estrogen is cardioprotective, because hormone replacement therapy reduces the risk of coronary artery disease in women.2,5–7 However, the mechanisms by which estrogen is cardioprotective are incompletely understood. Although estrogen lowers LDL cholesterol and increases HDL cholesterol,3,4 these changes account for only 75% of its cardioprotective effect in women.3,4 The remaining 25% is accounted for by alternative mechanisms.

Estrogen may be cardioprotective by enhancing vasodilation of the coronary circulation. Functional estradiol receptors are present on both endothelial4 and vascular smooth muscle cells.9,10 Acute infusions of estradiol increase coronary blood flow in the open-chest dog11 and the nonpregnant sheep.12,13 Several mechanisms have been proposed to mediate the direct effect of estradiol on the vasculature, including membrane-altering properties and/or calcium channel blocking,14 stimulation of the NO/cGMP pathway, and activation of K+ channels of the coronary vascular smooth muscle.11,15,16 Although it is important to understand how estradiol relaxes vascular smooth muscle directly, it may be independent of the genomic effect chronic estradiol administration has on the coronary vasculature.

Chronic exposure to estradiol has been shown to upregulate gene expression of endothelial NO synthase17–20 and thereby can increase NO production. We have previously shown that chronic 17β-estradiol replacement both increases calcium-dependent NO synthase activity of the guinea pig heart20 and decreases constrictor responses of isolated coronary arteries to the thromboxane mimetic U46619.21 The attenuated constrictor response with estradiol replacement was abolished by nitro-L-arginine (LNA), suggesting an enhanced basal NO production by estradiol.21 The effects of chronic estradiol on coronary responses are both time- and dose-dependent.20,21 Others have shown an attenuating effect.
of long-term estradiol administration on coronary tone in both humans\(^\text{22,23}\) and sheep\(^\text{12,13}\) that is inhibited by LNA.\(^\text{12}\) Chronic estradiol administration has also been shown to enhance agonist-stimulated vasodilation by acetylcholine in the coronary circulation of monkeys.\(^\text{24}\) In isolated arteries, estradiol given to rabbits for 4 days increased the endothelium-dependent relaxation to acetylcholine of aortic rings\(^\text{25,26}\) and of pig coronary artery rings incubated overnight in 17β-estradiol.\(^\text{27}\) In cultured guinea pig coronary smooth muscle cells, estradiol decreased the bradykinin-stimulated increase in intracellular calcium concentration.\(^\text{28}\) Ovariectomy has been shown to cause supersensitivity to constrictor agonists of isolated coronary artery smooth muscle cells of rhesus monkeys that was attenuated by chronic estradiol replacement.\(^\text{29}\) The present study investigated for the first time the effect of chronic estradiol replacement at various doses on agonist-stimulated vasodilation of the intact coronary microcirculation.

### Methods

The methods used were approved by University of Maryland Animal Care Committee.

#### Animal Preparation

Mature adult female guinea pigs (Hartley Strain; 500 to 600 g) were anesthetized with ketamine (80 mg/kg IM) and xylazine (1 mg/kg IM). Ovaries were removed bilaterally from all the animals through flank incisions under sterile surgical conditions. Animals were allowed to recover for 100 days to mimic more closely the effects of prolonged ovarian dysfunction, similar to that in postmenopausal women. Pellets (21-day release) containing 0.1, 0.25, 0.5, or 1.0 mg 17β-estradiol (Innovative Research of America) were then implanted subcutaneously in the back of the neck in anesthetized animals. A range of doses of estradiol was selected to ensure that the levels of circulating estradiol included a physiological range for the guinea pig. Control animals were ovariec tomized but did not receive estradiol.

#### Tissue Preparation

The hearts were removed from anesthetized animals 19 to 20 days after placement of pellets. Release of estradiol from the pellet is reported to be linear, following zero-order kinetics up to 21 days, according to the manufacturer’s recommendations. Heparinized saline was first injected into the vena cava to prevent clot formation, and the hearts were then rapidly excised through a thoracotomy and immediately weighed in ice-cold oxygenated Krebs-bicarbonate buffer containing the following (in mmol/L): NaCl 118, KCl 4.7, MgSO\(_4\)·7H\(_2\)O 1.18, KH\(_2\)PO\(_4\) 1.18, d-(+)-glucose 5.55, sodium pyruvate 2, Na-EDTA 0.016, NaHCO\(_3\) 15.8 (pH 7.35 to 7.4), and CaCl\(_2\)·2H\(_2\)O 2.2. Hearts were then mounted onto a 1.6-mm glass cannula of a perfused heart apparatus (Radnoti Glass Technology, Inc) at the base of the aorta and retrogradely perfused with oxygenated (95% O\(_2\)/5% CO\(_2\)) buffer. Contractile force was measured by inserting a surgical steel hook into the apex of the left ventricle and connecting it to a force transducer (Grass FT03 transducer). Hearts were stretched to a previously determined optimal contractile force and equilibrated for 30 minutes before the experiment was begun. They were then paced electrically at 235 bpm with platinum electrodes inserted into the ventricles (Grass model SD9 stimulator; stimulus parameters 1.4 ms, 3 V) and perfused with buffer at a constant flow rate adjusted to produce a perfusion pressure of 50 mm Hg. Perfusion pressure was measured by an inline Radnoti pressure transducer and recorded on a Grass model 7E Polygraph.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Serum Level, pg/mL</th>
<th>n</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 mg pellet</td>
<td>3.9±0.3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>0.1 mg</td>
<td>6.9±0.8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>0.25 mg</td>
<td>23.9±3.3</td>
<td>8</td>
<td>*</td>
</tr>
<tr>
<td>0.5 mg</td>
<td>28.3±6.0</td>
<td>8</td>
<td>*</td>
</tr>
<tr>
<td>1.0 mg</td>
<td>74.9±14.2</td>
<td>3</td>
<td>*</td>
</tr>
<tr>
<td>Nonpregnant</td>
<td>4.1±0.4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>16.0±2.4</td>
<td>4</td>
<td>*†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Pellets containing various mg amounts of 17β-estradiol are indicated above. n refers to the number of guinea pigs.

### Radioimmunoassay for 17β-Estradiol

Arterial blood samples were collected from the aorta in polypropylene tubes and centrifuged at 4000 rpm at 4°C. The serum supernatant was placed in a second set of polypropylene tubes and stored at −20°C until assayed. Serum 17β-estradiol was measured by the Coat-A-Count methodology (Diagnostics Products Corp) according to the manufacturer’s instructions.

#### Experimental Protocol

Vasodilator responses of isolated constant-flow-perfused hearts were measured as a change in perfusion pressure. The coronary circulation was initially constricted with prostaglandin (PG) F\(_2\)\(_\alpha\) (1 μmol/L), and dilator responses to cumulative addition of acetylcholine (10\(^{-8}\) to 10\(^{-5}\) mol/L) and sodium nitroprusside (10\(^{-4}\) to 10\(^{-3}\) mol/L) were measured. PGF\(_2\)\(_\alpha\) and either of the vasodilator agonists were infused into the tubing, perfusing the aorta via a Harvard syringe pump at a rate selected for the desired concentration. After each dose-response curve, there was a 30-minute washout period until perfusion pressure returned to baseline. Both acetylcholine and sodium nitroprusside were infused consecutively into the same heart and then subsequently repeated in the presence of LNA (10\(^{-5}\) mol/L). Because LNA increased the perfusion pressure, the concentration of PGF\(_2\)\(_\alpha\) infused was adjusted to match the same perfusion pressure as achieved under control conditions. Time controls demonstrated no significant differences between consecutive dose-response curves to either vasodilator.

### Statistical Analysis

Vascular resistance (mm Hg · mL\(^{-1}\) · min\(^{-1}\) · g heart\(^{-1}\)) was calculated as the perfusion pressure (mm Hg) divided by the flow rate (mL · min\(^{-1}\) · g heart\(^{-1}\)). Vasodilation was normalized as a percentage of the maximal response to each agonist achieved at 10\(^{-5}\) mol/L for acetylcholine and sodium nitroprusside. The −log EC\(_50\) value (the negative log concentration producing 50% of maximal vasodilation) was used as an index of agonist potency. Myocardial contractile force (g/g heart wt) was normalized to the heart weight. Dilator responses were compared between untreated (control) and estradiol-treated animals by 2-way repeated-measures ANOVA with calculated vascular resistance as the dependent variable and LNA treatment and estradiol dose as independent variables. If the mean values for the ANOVA were found to be statistically significant (P<0.05), a Newman-Keuls test was applied to analyze differences between treatments. Estradiol serum levels were analyzed by Kruskal-Wallis ANOVA followed by Dunn’s method of multiple comparison testing for unequal n values.

### Results

Serum 17β-estradiol levels were measured in the pelleted and ovariectomized guinea pigs and compared with both intact nonpregnant and pregnant guinea pigs (Table). Serum 17β-
estradiol concentrations in animals given 0.25-, 0.5-, and 1.0-mg pellets were significantly greater than those in ovariectomized controls and animals given 0.1-mg pellets. Non-pregnant guinea pigs had serum 17β-estradiol levels (4.1±0.4 pg/mL; n=8) similar to those of ovariectomized controls (3.9±0.3 pg/mL; n=8). All samples were run in a single assay, with intra-assay variation of 7.8%.

Both uterine and heart weights normalized to total body weight (which were similar between groups) were increased significantly compared with the respective organ weights of untreated ovariectomized controls (Figure 1). Despite no significant increase in serum estradiol levels, a 0.1-mg dose of 17β-estradiol increased uterine weight significantly, by 4.5-fold compared with uteri of ovariectomized controls and 1.8-fold compared with uteri of intact nonpregnant guinea pigs. Uterine weight was not further increased in animals given pellets containing 0.25, 0.5, or 1.0 mg 17β-estradiol.

Baseline coronary resistance was similar among the 5 animal groups (8.8±0.2, 8.7±0.2, 8.4±0.3, 8.5±0.4, and 8.6±0.4 mm Hg · min⁻¹ · g heart⁻¹ for 0.0, 0.1, 0.25, 0.5, and 1.0 mg 17β-estradiol, respectively). LNA infusion alone increased calculated coronary resistance in ovariectomized controls significantly, by 15.9%. There was a further increase of 41.4%, 29.8%, 34.1%, and 41.4% in hearts from guinea pigs treated with 0.1, 0.25, 0.5, and 1.0 mg 17β-estradiol, respectively. Under control conditions, infusion of PGF₂α (1 μmol/L) increased coronary vascular resistance by ≈1.7-fold in all groups. In the presence of LNA, the perfusion pressure was matched, before the start of either acetylcholine or sodium nitroprusside infusion, by reducing the PGF₂α concentration to ≈0.3 μmol/L. Thus, there were no significant differences in the calculated coronary resistance in hearts perfused with PGF₂α before or after treatment with LNA.

The cumulative addition of acetylcholine caused a dose-dependent decrease in perfusion pressure and calculated coronary vascular resistance. Responses were normalized to the vasodilator response at 10⁻³ mol/L acetylcholine (Figure 2). 17β-Estradiol, at all doses, caused a progressive leftward shift in the dose-response curve to acetylcholine compared with hearts from untreated ovariectomized animals (Figure 2). Negative log EC₅₀ values for acetylcholine were significantly increased with all estradiol doses ≥0.1 mg (Figure 3, top), and values at 1.0 mg were significantly greater than those at 0.1 mg estradiol. Maximal relaxation to acetylcholine, measured as coronary resistance at 10⁻² mol/L acetylcholine, was similar among all estradiol-treated groups compared with untreated ovariectomized controls (Figure 3, bottom). In the presence of LNA, maximal resistance to 10⁻³ mol/L acetylcholine was increased compared with control, although there were no significant differences among the groups (Figure 3). Furthermore, LNA decreased −log EC₅₀ values in all groups and shifted the dose-response curves to
the right compared with their respective untreated controls. Finally, LNA abolished differences in $-\log EC_{50}$ values among estradiol-treated groups (Figures 2 and 3).

The cumulative addition of sodium nitroprusside caused a dose-dependent vasodilation (Figure 4). However, there was no significant effect of chronic $17\beta$-estradiol or LNA on either maximal resistance or $-\log EC_{50}$ values to sodium nitroprusside (Figures 4 and 5). Although the dose-response curves did not reach a plateau at the highest dose, the maximal response to $10^{-3}$ mol/L sodium nitroprusside was only 5% to 10% less than that to $10^{-5}$ mol/L papaverine, an agonist used to determine maximal coronary vasodilation. The maximal vasodilation to $10^{-3}$ mol/L sodium nitroprusside, in experiments tested, was similar to that achieved with $10^{-5}$ mol/L papaverine (not shown).

Myocardial contractile force was measured to assess the stability of the preparation and myocardial contractility. Overall, there were no significant differences among the groups in the contractile force normalized to heart weight with respect to dose of estradiol or LNA infusion (data not shown). Furthermore, contractile force remained steady during the duration of the dose-response curve for both dilators. However, there was a consistent decrease ($\approx 10\%$) in force at $10^{-5}$ mol/L for acetylcholine that was not statistically significant.

**Discussion**

Chronic estradiol replacement increases the sensitivity of the guinea pig coronary microcirculation to acetylcholine, an endothelium-dependent vasodilator, but not to the NO donor sodium nitroprusside. LNA abolished the estradiol-induced increase in vasodilation, suggesting a stimulating effect of chronic estradiol on agonist-induced NO production in the heart. The lack of effect of estradiol on sodium nitroprusside–induced vasodilation suggests that NO sensitivity of the coronary resistance-sized arteries is unaltered by chronic estradiol replacement. Furthermore, we have previously shown increased calcium-dependent NO synthase activity with estradiol replacement in the guinea pig heart. Thus, chronically administered estradiol at doses considered physiological may enhance vasodilator responses to acetylcholine through enhanced NO production from the coronary endothelium.

The present study provides new evidence that the coronary microcirculation is an important target site of estrogen replacement therapy for potentiating agonist-stimulated vasodilation. Acetylcholine dilates the guinea pig coronary circulation by stimulating the release of endothelium-derived NO. In the present study, LNA significantly inhibited the maximal vasodilator response to acetylcholine but not sodium nitroprusside, suggesting the release of NO by acetylcholine. However, the remaining acetylcholine-induced coronary vasodilation in the presence of LNA suggests the contribution of other NO-independent factors, such as vasodilator prosta-
The heart is increased with chronic estradiol replacement after 10 days. Estradiol replacement had no significant effect on baseline coronary resistance in the present study. However, the constrictor effect of LNA was greater in hearts from estradiol-treated guinea pigs than in the ovariectomized controls, suggesting an estradiol-dependent increase in basal NO production. In a separate study using isolated constant-pressure-perfused rabbit hearts, 17 β-estradiol replacement for 4 days increased baseline coronary flow compared with untreated controls, which was inhibited in the presence of LNA. Both studies support an enhanced basal NO production by hormone replacement, although differences in baseline flow responses to estradiol may reflect differences between species, the duration of replacement, and/or constant-flow versus constant-pressure perfusion.

Estradiol enhances acetylcholine sensitivity of the coronary microcirculation at a dose of estradiol (0.1 mg) that did not alter heart weight. This suggests that the coronary vascular bed may be more sensitive to estradiol replacement than myocardial cells. Furthermore, there was a gradual increase in acetylcholine sensitivity with increasing doses of estradiol, which was abolished in the presence of NO inhibition. It is important to note that although differences between the dose-response curves were small, there was a significant difference in the acetylcholine sensitivity between 0.1 and 1.0 mg estradiol, suggesting a dose dependency. Previous studies have demonstrated a supersensitivity to constrictor agents after ovariectomy that was normalized with estradiol replacement. This suggests that low-dose estradiol enhances the sensitivity to agonist-stimulated NO release from the coronary microcirculation. Thus, estradiol replacement, either experimentally or in postmenopausal women, may inhibit the hyperreactivity to constrictor substances by increasing the inhibitory benefit of endothelium-derived NO from the coronary circulation, thereby preventing coronary vasospasm. Furthermore, estradiol replacement has a selective effect on endothelium-dependent mechanisms, whereas vascular smooth muscle sensitivity to NO of the coronary microcirculation is unaffected.

Chronic estradiol replacement increased both uterine and heart weights. However, there was a significant difference in estradiol sensitivity between the 2 organs. Uterine weight increased significantly at the lowest dose of estradiol (0.1 mg) tested compared with the ovariectomized control and did not increase further at higher doses. Similarly, in ovariectomized rats, uterine weight was also maximally increased at the lowest dose of estradiol tested (0.1 mg) and did not increase further with higher doses (0.5 to 50 mg; timed-release pellets placed subcutaneously). It has been shown previously that in both nonpregnant and pregnant guinea pigs, uterine estradiol content is ~10-fold greater than plasma estradiol concentration, suggesting a large difference between plasma levels and uterine content. This is attributed to the relatively high concentration of estradiol receptors found in this tissue. It has been shown previously that in both nonpregnant and pregnant guinea pigs, uterine estradiol content is ~10-fold greater than plasma estradiol concentration, suggesting a large difference between plasma levels and uterine content. This is attributed to the relatively high concentration of estradiol receptors found in this tissue.

Importantly, the LNA-insensitive vasodilation was unaffected by chronic estradiol replacement, suggesting that the NO-dependent but not -independent vasodilator mechanisms are estradiol-sensitive. In previous studies, chronic estradiol replacement was also shown to attenuate the constrictor response of the coronary circulation to acetylcholine in postmenopausal women. In large-diameter arteries, chronic estradiol treatment enhances acetylcholine- but not sodium nitroprusside–stimulated relaxation of the isolated rabbit aorta and pig coronary arteries. We report a similar finding in the coronary resistance arteries of the guinea pig heart.

We propose that chronic estradiol enhances agonist-stimulated coronary microvascular dilation via upregulation of NO synthase transcription. We have previously shown that calcium-dependent NO synthase activity of the guinea pig heart is increased with chronic estradiol replacement after 10 days. An estrogen-induced increase in endothelial NO synthase mRNA has been shown in other cell types, such as fetal pulmonary artery endothelium, uterine artery endothelium, human endothelial EA.hy 926 cells, and skeletal muscle. Further study is needed to determine the specific cell types in which estradiol induces upregulation in the intact guinea pig heart. Alternatively, acetylcholine receptor expression can be variably altered with estradiol treatment, depending on the tissue type. However, Bell et al demonstrated in isolated pig coronary arteries that estradiol treatment also enhanced relaxation to A23187, suggesting that the endothelium-dependent relaxation is enhanced independently of muscarinic receptors.

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It is expected in the present study that uterine estradiol content would also be high even at the relatively low plasma concentrations measured. We speculate that uterine estradiol content may be maximal at the lowest plasma estradiol levels to explain the maximal growth response. Furthermore, the heart may require a higher plasma concentration than the uterus to evoke a growth response, because the guinea pig uterus has been shown to have a higher estradiol content than several other nonreproductive organs, including the heart.29 Thus, low-dose estradiol replacement may be beneficial in selectively enhancing endothelium-dependent responses of the coronary circulation independently of inducing estrogen-mediated growth-promoting effects in the heart as well as other nonreproductive organs.

It remains unclear why serum estradiol concentration did not exhibit a classic dose-dependent response with pellets of increasing estradiol amounts. Our values are similar to those of previous studies having serum estradiol concentrations of guinea pigs ranging from undetectable to 22 pg/mL in nonpregnant guinea pigs to 22 to 50 pg/mL in pregnant guinea pigs.8,35,37 Because guinea pigs were allowed 100 days to recover from the castration, differences in body fat content and size may contribute to the differences in the ability of the guinea pig organs to retain circulating estradiol. Castration leads to an increase in adipose tissue and the production of adrenal androgen precursors. The lack of a decline in estradiol levels after castration most likely reflects peripheral aromatization. The difficulty in correlating absolute serum estradiol levels with tissue-specific responses may be related to variable levels of serum binding proteins as well as differences in tissue retention. Regardless, our results show a progressive increase in vascular sensitivity to acetylcholine that correlates with increasing doses of estradiol.

We have previously shown that estradiol replacement inhibits U46619 (a thromboxane mimetic)-induced contraction of isolated guinea pig coronary arteries at lower doses of estradiol (0.25 and 0.5 mg) but enhances contractility at higher doses (1.5 and 7.5 mg), revealing a bell-shaped response to increasing estradiol.21 This was attributed to an estradiol-induced increase in NO release, because LNA abolished the differences in reactivity to the contractile agonist. In the present study, the pattern of response to chronic estradiol replacement differs (i.e., no loss of benefit with higher doses), probably reflecting differences in reactivity between large and resistance-sized arteries. The important similarity between the studies, however, was a beneficial effect at the lowest doses tested, whether enhancing the dilator sensitivity to acetylcholine in the coronary microcirculation or inhibiting the constrictor response of the main coronary artery. Thus, doses selected for hormone therapy, producing circulating levels of estradiol within a physiologic range, may be sufficient to produce beneficial cardiovascular effects.

In summary, chronic estradiol enhances endothelium-derived NO release from the coronary microcirculation, whereas NO sensitivity is unaltered. Chronic estradiol may enhance agonist-stimulated endothelium-derived NO produc-

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**References**


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