Nitric Oxide Accounts for Dose-Dependent Estrogen-Mediated Coronary Relaxation After Acute Estrogen Withdrawal

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**Background** Estrogen replacement therapy reduces the risk of coronary heart disease in postmenopausal women, and estrogen treatment modulates endothelium-dependent vasodilation in ovariectomized, atherosclerotic monkeys. Estradiol-17β also induces relaxation in isolated rabbit coronary arteries as well as cerebral basilar arteries. The estrogen concentrations required to induce such relaxation are in the pharmacological range (10⁻⁸ to 10⁻⁶ mol/L).

**Methods and Results** The present study was designed to test whether the sensitivity and specificity of the relaxing response of coronary vascular smooth muscle to exogenous estradiol-17β is dependent on the sex hormone status of the animal. In coronary artery rings contracted with PGF₂α (3 x 10⁻⁶ mol/L), estradiol-17β caused significant relaxation at a physiological estradiol concentration (10⁻⁶ mol/L), in coronary artery rings from oophorectomized, estrogen-treated and acutely estrogen-withdrawn rabbits only. Relaxation induced by estradiol-17β at lower concentrations (10⁻⁸ to 10⁻⁶ mol/L) in these rings was 20±6%, 42±8%, 54±9%, and 76±8%, respectively, compared with 4±2%, 12±5%, 16±7%, and 25±12% and 5±2%, 12±5%, 18±8%, and 23±10% in rings from estrogen-maintained and oophorectomized rabbits, respectively (P<.01). The relaxation in coronary artery rings from estrogen-treated and acutely estrogen-withdrawn rabbits was endothelium and nitric oxide dependent since it was abolished by endothelium removal and the nitric oxide synthase inhibitor N⁶-nitro-L-arginine. 

**Conclusions** This study demonstrates that estrogen-induced, endothelium-dependent relaxation of coronary arteries in ovariectomized cynomolgus monkeys caused contraction, whereas in a group of similar animals treated with estradiol-17β, a relaxation response to acetylcholine was demonstrated. These findings have recently been confirmed in human coronary vessels. These in vivo studies suggest that estradiol-17β may be affecting vascular tone by an EDRF-dependent mechanism; however, the specificity of the acetylcholine response for EDRF was not tested. The possibility of the involvement of EDRF in vivo has been strengthened by evidence that estrogen-induced increases in flow in the uterine artery can be antagonized by nitric oxide synthase inhibition.

**Key Words** estrogen • nitric oxide • smooth muscle

Women appear to be protected until the menopause from the development of coronary artery syndromes. This protective effect appears to be due to the beneficial effect of ovarian hormones, estradiol-17β in particular, since estrogen replacement therapy reduces the incidence of coronary artery disease and the progression of coronary artery lesions. A number of potential mechanisms for the protective effect of estrogen on coronary arteries have been proposed. Estrogen has a beneficial effect on plasma lipoproteins, increasing high-density lipoprotein cholesterol and decreasing low-density lipoprotein cholesterol. While early studies suggested that over 50% to 60% of the protective effect of estrogens on coronary artery disease was due to the favorable change in plasma lipids, more recent data suggest that this figure is closer to 25%. Estrogens also appear to inhibit cholesterol deposition in the arterial wall. Other potential protective mechanisms of estrogen action include calcium antagonism, hormone-induced release of endothelium-derived relaxing factor(s), and suppression of contracting factors.

Estrogen increases cardiac output and arterial flow velocity and decreases vascular resistance and systolic and diastolic blood pressures. It relaxes precontracted coronary artery rings and inhibits calcium influx in isolated cardiac myocytes. A recent study demonstrated that estradiol-17β improves exercise-induced myocardial ischemia in postmenopausal women with angiographically proven coronary artery disease.

Acetylcholine induces endothelium-dependent vascular relaxation in vitro by the release of endothelium-derived relaxing factor (EDRF). The infusion of acetylcholine into atherosclerotic coronary arteries of ovariectomized hypercholesterolemic cynomolgus monkeys caused contraction, whereas in a group of similar animals treated with estradiol-17β, a relaxation response to acetylcholine was demonstrated. These findings have recently been confirmed in human coronary vessels. These in vivo studies suggest that estradiol-17β may be affecting vascular tone by an EDRF-dependent mechanism; however, the specificity of the acetylcholine response for EDRF was not tested. The possibility of the involvement of EDRF in vivo has been strengthened by evidence that estrogen-induced increases in flow in the uterine artery can be antagonized by nitric oxide synthase inhibition.

We therefore tested the hypothesis that the sex hormone status of the animal in vivo influences the responsiveness of the rabbit coronary artery in vitro to exogenous estrogen by eliciting relaxation responses to estradiol-17β in an organ bath.
Methods

Animals and Procedures

Sixteen New Zealand White female rabbits weighing 1.7 to 2.5 kg were assigned to three treatment groups (N=number of animals, n=number of ring segments; at least one ring segment was taken from each animal). Group 1 animals were oophorectomized, untreated female rabbits (N=5, n=6); group 2 animals were oophorectomized, estrogen-replaced female rabbits (N=5, n=17); and group 3 animals were oophorectomized estrogen rabbits with estrogen replaced and then estrogen withdrawn for 48 hours (N=6, n=14). The rabbits in all groups underwent transabdominal bilateral oophorectomy, using general anesthesia with ketamine (40 mg/kg IM), xylazine (4 mg/kg IM), and halothane, 10 to 15 days before treatment. Group 1 rabbits underwent oophorectomy followed by the insertion of a 2-cm estradiol vehicle (placebo)-filled silicon capsule. Estrogen replacement was instituted in group 2 by subcutaneously inserting a 2-cm estradiol-17β-filled silicon capsule. The subcutaneous capsules release 2 μg of estradiol within a 24-hour period. The estradiol-17β capsules were maintained in these rabbits for 9 days, until the day of the experiment. In group 3 rabbits, estrogen replacement and acute withdrawal was stimulated by removing the capsules on the 7th day of treatment, 48 hours before the experiment.

Preparation of Rabbit Coronary Arteries

The rabbits were killed on day 9 with an overdose of intravenous pentobarbital (60 mg/kg) after an intravenous injection of heparin (150 U/kg), in accordance with institutional guidelines. The hearts were removed and immediately immersed in ice-cold modified Krebs' solution containing (mmol/L) NaCl, 118.3; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.2; KPO4, 1.2; and glucose, 11.1. Indomethacin (10-5 mol/L), an inhibitor of prostanoetid synthesis, was added to the incubation medium. The left anterior descending or left circumflex coronary artery was dissected free of connective tissue. Rings (2- to 3-mm length) were prepared, and the presence of endothelium was verified by the relaxation in response to substance P (10 mmol/L).

Measurement of Arterial Tension

Coronary artery rings were suspended in organ baths and tension measured as previously described. They were suspended horizontally between two stainless steel hooks for measurement of isometric tension in individual organ baths containing 10 mL modified Krebs' solution at 37°C, bubbled with 95% O2 and 5% CO2. Rings were equilibrated for 45 minutes under a resting tension of 1 g before drugs were added.

Relaxing Effect of 17β-Estradiol

Coronary arterial rings were contracted with prostaglandin F20 (PGF2α, 3×10-5 mol/L), which represented the EC50. The integrity of the endothelium was determined by the relaxing response to substance P in every ring. Estradiol-17β (10-8 to 5×10-5 mol/L) or equivalent ethanol solvent (1:2000) was added 7 minutes after the addition of the constrictor agent, when the plateau of constriction was attained. The ability of the rings to relax to the endothelium-dependent relaxing agent acetycholine (10-8 to 10-4 mol/L) was determined, as was the ability of the rings to relax to the endothelium-independent relaxing agent sodium nitroprusside (10-7 to 10-2 mol/L).

Effect of N-Nitro-l-Arginine on Relaxation Induced by Estradiol-17β

N-nitro-l-arginine (L-NA) is an inhibitor of endothelium-derived nitric oxide synthesis from l-arginine in vascular endothelial cells. To determine whether any relaxing response to estrogen in any group was due to endothelium-dependent nitric oxide production, L-NA (3×10-5 mol/L) was preincubated 10 minutes before the addition of the constrictor agent in at least one ring per animal per group. Similarly, the endothelium was removed in at least one ring per animal per group. Estradiol-17β (10-8 to 5×10-5 mol/L) was added 7 minutes after the addition of the constrictor agent. Reversal of L-NA-induced inhibition of relaxation was attempted by the subsequent addition of L-arginine (10-4 mol/L).

Drugs

Estradiol-17β, acetylcholine, indomethacin, l-arginine, L-NA, sodium nitroprusside, PGF2α, and substance P were all supplied by Sigma.

Statistical Analysis

Values are expressed as mean±SEM. Relaxation is expressed as percentage of contraction. Comparisons of means were made by using the Student’s t test for unpaired values; when more than two means were compared, an ANOVA with repeated measurements was used. If a significant F value was found, Scheffé’s test for multiple comparisons was used to identify differences among groups. Statistically significant differences were assumed at the 5% confidence level.

Serum estradiol levels were measured by direct radioimmunoassay in every rabbit in each of the three groups. Due to the skewed distribution of the results, statistical comparisons were done using the natural logarithm of the results. Comparisons based on repeated measurements on rabbits used paired t tests, and comparisons of independent groups used two-sample t tests.

Results

Relaxing Effect of 17β-Estradiol on Precontracted Coronary Arteries

While PGF2α (3×10-5 mol/L) induced comparable submaximal contraction in rings from all groups of rabbits, there was an increased sensitivity in the relaxation response to estradiol-17β in coronary arterial rings from group 3 (oophorectomized rabbits that were estrogen replaced and then acutely hormone deprived for 48 hours). Estradiol-17β (10-4 to 10-6 mol/L) induced significantly greater relaxation only in rings from this group of animals (P<0.01; Fig 1). Estradiol-17β (10-5 and 5×10-5 mol/L) induced significant concentration-related relaxation of contracted rings in all three rabbit groups (compared with time-matched ethanol solvent controls, all P<0.01; Fig 1). Relaxing responses to acetylcholine (10-8 to 10-5 mol/L) and sodium nitropruss-
side (10⁻⁷ to 10⁻⁵ mol/L) were no different in any group (Table).

**Effect of L-NA, Endothelium Removal, and Indomethacin on Estradiol-17β–Induced Relaxation**

L-NA (5×10⁻⁶ mol/L) significantly inhibited the relaxation induced by the smaller concentrations of estradiol-17β (10⁻⁹ to 10⁻⁵ mol/L, Fig 2) in the rings from group 3 animals (by 71±11%, 72±11%, 72±12%, and 69±13%, respectively, P<.05) but did not inhibit the relaxation induced by greater concentrations of estradiol-17β (10⁻³ and 5×10⁻⁷ mol/L by 29±12% and 10±5%, respectively, P=NS). Indomethacin (10⁻⁵ mol/L) had no effect on the relaxation induced by estradiol-17β (10⁻⁹ to 10⁻⁶ mol/L) in the rings from group 3 animals or the relaxation induced by the higher concentrations of estradiol-17β (10⁻⁵ and 5×10⁻⁵ mol/L) in rings from all three groups. In group 3 animals, endothelium removal had the same effect on the relaxing responses to estrogen (10⁻⁹ to 10⁻⁴) as L-NA (7±3%, 15±2%, 17±3%, and 17±3% versus 4±3%, 6±4%, 8±3%, and 18±4%, respectively; P=NS). L-NA–induced inhibition was reversed by L-arginine by 54±8% (10⁻¹ mol/L; N=5, see Fig 3 for representative trace).

**Serum Estradiol Levels**

In group 1 (oophorectomized), the serum estradiol concentration was 12.26±2.31 pg/mL. In group 2 (estrogen maintained), the serum concentration was 39.63±1.32 pg/mL, and in group 3 (acutely withdrawn), the serum estradiol concentration was 10.83±3.41 pg/mL. There was a significant decrease in serum estradiol levels in animals in group 3 at 48 hours after the withdrawal of the estrogen capsules (P<.001, two-sample t test), and this level was similar to the level in group 1 animals (P=NS, two-sample t test).

**Discussion**

In this study we confirm our previous findings that estrogen directly relaxes vascular myocytes at a concentration of 10⁻⁵ mol/L. Additionally, this study provides evidence for an important endothelium-mediated relaxation, dependent on the preexisting sex hormone status of the animal. There was an observed significant difference in the relaxing sensitivity of rings from rabbits in group 3 (oophorectomized, estrogen replaced, and then acutely hormone deprived for 48 hours). L-NA abolished this relaxation. It appears that the acute estrogen withdrawal is in some way enabling exogenous estradiol-17β to facilitate the production of, or the response to, nitric oxide produced by the endothelium. The mechanism of this effect is undetermined, but the similar response to sodium nitroprusside in rings from both

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**Table**

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<tr>
<td><strong>Group 1</strong></td>
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Statistical significance was determined between groups for each concentration of the agent by an unpaired Student’s t test.
groups would suggest that the mechanism lies at the level of the production of nitric oxide from the endothelial cells. The mechanism may also be distinct from the muscarinic receptor-linked nitric oxide pathway, since the response to acetylcholine was no different in rings from the different animal groups. This relaxation differs from that observed in the two other groups in that it occurs at concentrations three orders of magnitude less. To our knowledge, this represents the first demonstration of an acute relaxation effect of estrogen in the physiological range in vitro. The time course of this effect suggests a membrane-mediated effect. The relaxation caused by estrogen in low concentrations in these animals is abolished by L-NA, and this abolition is overcome by L-arginine. These observations strongly suggest that the endothelial cell can be stimulated by estrogen to synthesize and secrete nitric oxide under certain hormonal conditions, in this case, acute estrogen deprivation.

Evidence that estradiol-17β has direct relaxing properties in the coronary artery in vitro has been reported by Jiang et al.,17,18 who demonstrated that estradiol-17β induced an equal degree of relaxation in rabbit coronary arteries with and without endothelium. A calcium-antagonistic property of estradiol-17β was suggested in isolated coronary arterial preparations and confirmed in cardiac myocyte preparations using electrophysiological techniques.17,20 We have also shown that acute estrogen and progesterone withdrawal in vivo increases cerebral vasoreactivity to serotonin in rabbit basilar artery segments through an endothelium-independent mechanism.25

Femoral arteries from rabbits treated with estradiol-17β show an enhanced endothelium-dependent relaxation to acetylcholine.13 However, this enhanced endothelium-dependent relaxation to acetylcholine was seen only at lower concentrations (3 × 10⁻⁹ to 3 × 10⁻⁸ mol/L). The endothelium-dependent responses to the calcium ionophore A23187 were unchanged, suggesting a peculiarity to acetylcholine only. In another study,14 despite an increased basal release of nitric oxide in endothelium-intact aortic rings from female rabbits over those from males, the relaxation responses to acetylcholine (3 × 10⁻⁹ to 3 × 10⁻⁵ mol/L), A23187, and nitroglycerin were identical in rings from male, ovary-intact female, and oophorectomized female rabbits. The similarity of these data and ours may be the result of physiological concentrations of estradiol-17β, whereas supraphysiological concentrations of estradiol-17β were used in the study by Gisclard et al.15 It has also been demonstrated that estrogen-induced increases in blood flow in the uterine artery can be antagonized by nitric oxide synthase inhibition.15 Recent reports demonstrate that long-term (2 years, oral)23 and short-term (20-minute intravenous infusion)27 ethyl estradiol treatment modulates responses to acetylcholine in coronary arteries of cynomolgus monkeys and humans,24 suggesting a possible endothelium-dependent effect in vivo. We have observed that rabbit basilar arteries may demonstrate both endothelium-dependent and -independent estrogen relaxation, the former being dependent on the sex hormone status of the animal.25,28

This study indicates that estradiol-17β may modulate coronary vascular tone by a nitric oxide–dependent mechanism in the acutely estrogen-withdrawn animals. The acutely estrogen-withdrawn animals closely resemble the acute human menopausal state or the postmenopausal woman who has her hormonal supplements acutely withdrawn. The acute vasomotor disturbances that are observed at this time could be partially explained by an acute impairment of estrogen-dependent nitric oxide production. Recent work suggests that estrogen can stimulate constitutive nitric oxide synthase in cultured bovine endothelial cells.29 This effect in vitro, however, is not acute and takes 16 to 24 hours. There are also conflicting data showing no effect on nitric oxide synthase in cultured bovine aortic endothelial cells.30 An acute effect on the constitutive enzyme in fresh noncultured endothelium cannot be ruled out, and an acute effect on the inducible enzyme is possible. The interesting observation that the acute application of estrogen to coronary arterial rings from chronically oophorectomized animals did not result in relaxation at the lower estrogen concentrations may suggest that in these animals, there is a more permanent disruption of the estrogen–nitric oxide axis. An alternative explanation could be an upregulation of estrogen receptors on the endothelium in response to the sudden withdrawal of the circulating estrogen. This mechanism would assume that estrogen receptors, classic or otherwise, are involved in estrogen-induced nitric oxide production.

Whatever the mechanism, acute estrogen withdrawal could have important clinical implications, since sex hormone withdrawal associated with the menopause substantially increases the incidence of myocardial infarction and coronary atherosclerotic disease in postmenopausal women.31 An increased sensitivity to vasoconstrictor stimuli due to a lack of hormone-facilitated production of nitric oxide could play a pathophysiological role in acute coronary syndromes in postmenopausal women with atherosclerotic coronary heart disease.

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**References**

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