Effect of 17β-estradiol in hypercholesterolemic rabbits with severe endothelial dysfunction

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Do Nascimento, Carlos Antonio, Katalin Kauser, and Gabor M. Rubanyi. Effect of 17β-estradiol in hypercholesterolemic rabbits with severe endothelial dysfunction. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1788–H1794, 1999.—17β-Estradiol prevents early vascular lesion development and may also affect advanced atherosclerosis. To test the antiatherosclerotic effect of estrogen under conditions that resemble more advanced human atherosclerosis with severe endothelial dysfunction, we have investigated the effect of 17β-estradiol in hypercholesterolemic rabbits treated with the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME). Chronic L-NAME administration attenuated endothelial nitric oxide (EDNO)-mediated vascular responses leading to significantly accelerated atherosclerotic plaque development. 17β-Estradiol treatment alone inhibited aortic lesion formation with concurrent increase in EDNO-mediated responses. The beneficial effect of estrogen persisted in the L-NAME-treated rabbits, suggesting that the antiatherogenic action of 17β-estradiol involves NO-independent mechanisms as well. Serum cholesterol levels were not altered by any of the treatments. 17β-Estradiol treatment significantly increased EDNO production under these conditions as well. The reduction in plaque size by 17β-estradiol was always accompanied by increased EDNO production, suggesting a strong association between these two events. The results demonstrate that estrogen treatment may exert protection against atherosclerosis even in patients with severe endothelial dysfunction.

The antiatherogenic properties of 17β-estradiol have been well documented in epidemiological and clinical investigations as well as in animal studies, including the hyperlipidemic rabbit (19, 23). These studies suggest that beyond its lipid-lowering effect, 17β-estradiol has direct vascular effects, and the vascular endothelium may be a target of its action leading to cardiovascular protection. Because of its beneficial cardiovascular actions, EDNO seems to be an ideal mediator of the antiatherosclerotic effect of 17β-estradiol. Indeed, up-regulation of NO synthase (NOS) III in response to estrogen treatment has been reported both in vitro (13, 14, 20) and in vivo (9, 28). Therefore, it is conceivable that 17β-estradiol treatment can exert cardiovascular protection even in stages of atherosclerosis with severe endothelial dysfunction.

To test this hypothesis, the present study was designed to investigate the effect of 17β-estradiol in ovariectomized, hypercholesterolemic rabbits in which severe endothelial dysfunction, diminished EDNO-mediated vasorelaxation, was induced by chronic inhibition of NOS.

METHODS

Animals

Thirty-two female New Zealand White rabbits, weighing 3–3.5 kg, were ovariectomized under ketamine anesthesia. After a 1-wk recovery period following surgery, animals were fed by 1% cholesterol-containing rabbit food. One week after the start of high-cholesterol feeding, rabbits were randomly selected into four treatment groups (each consisting of 8 animals): 1) vehicle control group (VC), receiving daily subcutaneous injections of 0.2 ml of benzyl benzoate-castor oil (1:9 mixture); 2) L-NAME group, provided with 100 µg/ml L-NAME in the drinking water; 3) 17β-estradiol group (E2), injected with daily 30 µg of 17β-estradiol dissolved in vehicle (0.2 ml); and 4) L-NAME + E2 group, supplied with 100 µg/ml L-NAME in drinking water and injected with daily 30 µg of 17β-estradiol. The animals were housed individually and provided daily with fresh drinking water, either with or without L-NAME, ad libitum. The dose of L-NAME was selected based on earlier reports (3, 22) and pilot studies using 50, 100, and 150 µg/ml L-NAME via subcutaneous administration to ovariectomized rabbits on normal food. Three rabbits were tested for each concentration. Inhibition of EDNO-mediated responses was not different between the 100 and 150 µg/ml L-NAME-treated group, and after 1-wk administration of the NOS inhibitor, no significant differences could be measured in mean arterial blood pressure of ketamine-anesthetized rabbits (data not shown). Water intake was monitored daily and weight gain was monitored weekly. Food intake of the animals was limited to daily 150 g of cholesterol-containing rabbit food. Under these controlled circumstances, there was no significant difference in weight gain, water intake, and total serum cholesterol level of the animals from the different

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treatment groups (Table 1). The study was conducted according to protocols approved by the Animal Care Committee at Berlex Biosciences, in agreement with the recommendation of the American Association for the Accreditation of Laboratory Animal Care.

Determination of Atherosclerotic Vascular Lesions

Animals were killed after 7 wk of treatment (8 wk on 1% cholesterol diet). At death, blood was collected to measure serum cholesterol and estrogen levels, and the thoracic aorta was dissected. The proximal part of the aorta, including the aortic arch, was fixed in 10% Formalin. After being cleansed from the adherent connective tissues, the aortas were cut open longitudinally and pinned down individually on silicon-coated petri dishes, intimal surface up. Atherosclerotic plaque areas (fatty lesions) were visualized by staining the aortas with oil-red O. Total and plaque-covered areas were quantified by image analysis of the scanned photographic pictures.

Endothelium-Dependent Vascular Responses

Four rings of the distal end of each thoracic aorta, from the same animals in which the plaque areas were determined, were immediately placed in cold physiological saline solution of the following composition (in mM): 141 NaCl, 125 Cl\(^{-}\), 2.5 Ca\(^{2+}\), 4.7 K\(^{+}\), 0.76 Mg\(^{2+}\), 1.7 H\(_2\)PO\(_4\), 25 HCO\(_3\), 0.026 EDTA, 11 glucose, and 5 HEPES. After the adherent connective tissues were cleaned off, the endothelium was removed in two of the four rings by rubbing the intimal surface with a cotton swab. The rings were then mounted in organ chambers (Schuler, Hugo Sachs Elektronik, March-Hugstetten, Germany), and changes of isometric tension in response to drug treatment were measured with force transducers (Grass FT03, Hugo Sachs) and recorded by a four-channel recorder (Graphitec Linearcorder WR 3310, Hugo Sachs). Successful removal of the endothelium was confirmed by the absence of relaxation in response to ACh (1 \(\mu\)M)-contracted rings. Rings with and without endothelium were studied in parallel. Basal EDNO production was estimated by measuring \(\text{N}^\text{G}\)\text{-nitro-L-arginine (L-NNA; 100 \(\mu\)M)}-induced, endothelium-dependent tension development in PE-contracted (EC\(_50\) = 3 \(\mu\)M) aortic rings (25). Stimulated EDNO release was estimated by dose-dependent endothelium-mediated relaxation evoked by ACh (10 nM-10 \(\mu\)M) in rings contracted with 3 \(\mu\)M PE. These studies were performed in the presence of 10 \(\mu\)M indomethacin to block the production of vasoactive prostanooids. Relaxation to nitroglycerin (NG; 1 nM-1 \(\mu\)M) was also investigated in rings without endothelium to test the effect of treatments on the sensitivity of the vascular smooth muscle to NO.

Plasma Cholesterol and 17\(\beta\)-Estradiol Determination

Blood samples were collected from the inferior vena cava during euthanasia. Plasma estradiol level was determined by radioimmunoassay (Cornell University, Ithaca, NY). Serum total cholesterol was measured at Consolidated Veterinary Diagnostics (West Sacramento, CA).

Materials

ACh chloride, NG, PE, \(\text{L}\)-NAME, and \(\text{L}\)-NNA were obtained from Sigma Chemical (St. Louis, MO). Fresh stock solutions, used for the contractility studies, were prepared freshly before each experiment. All stock solutions were made in distilled water, and further dilutions were made in physiological salt solution, except indomethacin, which was dissolved in Na\(_2\)CO\(_3\) (0.2 M) and distilled water (1:9). 17\(\beta\)-Estradiol was provided by Schering (Berlin, Germany). 17\(\beta\)-Estradiol was dissolved in benzyl benzoate and castor oil as a 1:9 mixture. This solution was prepared fresh every 2 wk during the study. Benzyl benzoate and castor oil were obtained from Sigma Chemical. 17\(\beta\)-Estradiol and its vehicle were administered as a subcutaneous injection in a 0.2-ml volume daily for 7 wk.

Calculations and Statistical Analysis

The concentration of PE causing half-maximal contraction (EC\(_{50}\)) was calculated from full dose-response curves. Relaxation evoked by ACh and NG is expressed as percent inhibition of the contraction by PE. Lesion area is expressed as a percentage of the total luminal surface of the thoracic aorta. Results are presented as means \(\pm\) SE for the number of experiments on different animals (n) indicated. Statistical analyses of the data were performed by ANOVA or Student’s t-tests for unpaired observations. Data were considered to be significantly different at a value of P < 0.05.

RESULTS

Atherosclerotic Vascular Lesions

Cholesterol feeding for 8 wk resulted in significant fatty streak formation in the thoracic aortas of all rabbits. In the VC group (n = 8), 76 \(\pm\) 8% of the aortic arch (Fig. 1A) and 30.2 \(\pm\) 4% of the luminal surface of the thoracic aorta (Fig. 1B) were covered with atheroma-tous plaques as determined by image analysis of the scanned photograph of the oil-red O-stained segments. Chronic \(\text{L}\)-NAME treatment (n = 8) significantly (P < 0.05) increased the lesion-covered areas to 95.2 \(\pm\) 3% in the aortic arch and to 47.3 \(\pm\) 8% in the thoracic aorta compared with controls (Fig. 1). 17\(\beta\)-Estradiol treatment (n = 8) significantly (P < 0.0001) inhibited the development of aortic lesions compared with both the VC and \(\text{L}\)-NAME groups. The calculated plaque-to-surface area ratio was 47.1 \(\pm\) 8% in the aortic arch and 41.1 \(\pm\) 1% in the thoracic aorta, respectively (Fig. 1). 17\(\beta\)-Estradiol administration to the \(\text{L}\)-NAME-treated rabbits (n = 8) resulted in significant decrease of the

Table 1. Body weight, water intake, serum total cholesterol, and serum 17\(\beta\)-estradiol values in hypercholesterolemic ovariectomized rabbits treated with vehicle, 17\(\beta\)-estradiol, and \(\text{L}\)-NAME alone and in combination

<table>
<thead>
<tr>
<th></th>
<th>VC</th>
<th>E(_2)</th>
<th>L-NAME</th>
<th>E(_2)+L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>3.42 (\pm) 0.09</td>
<td>3.85 (\pm) 0.05</td>
<td>3.50 (\pm) 0.17</td>
<td>3.40 (\pm) 0.11</td>
</tr>
<tr>
<td>Daily water intake, ml</td>
<td>362 (\pm) 106</td>
<td>343 (\pm) 134</td>
<td>325 (\pm) 118</td>
<td>371 (\pm) 143</td>
</tr>
<tr>
<td>Serum total cholesterol, mg/dl</td>
<td>2,370 (\pm) 168</td>
<td>2,118 (\pm) 210</td>
<td>2,388 (\pm) 240</td>
<td>2,208 (\pm) 186</td>
</tr>
<tr>
<td>Serum 17(\beta)-estradiol, pg/ml</td>
<td>15.6 (\pm) 0.6</td>
<td>107.9 (\pm) 20.3*</td>
<td>10.8 (\pm) 1.3</td>
<td>123.7 (\pm) 20.6*</td>
</tr>
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Values are means \(\pm\) SE of 8 experiments in each group. VC, vehicle; E\(_2\), 17\(\beta\)-estradiol; \(\text{L}\)-NAME, \(\text{N}\)\(^{\text{G}}\)-nitro-L-arginine methyl ester.

*Significantly different from VC and \(\text{L}\)-NAME.
plaque-covered areas in both the aortic arch (57.1 ± 8%) and the thoracic aorta (10 ± 2%) compared with vessels isolated from the L-NAME-treated animals (Fig. 1). The plaque-to-surface area ratio was not statistically different from the E2 group.

The percent reduction in plaque size by 17β-estradiol treatment was not significantly different in the absence (aortic arch, 20.5 ± 10%; thoracic aorta, 74 ± 26%) and presence of L-NAME treatment (aortic arch, 27.1 ± 7%; thoracic aorta, 60.1 ± 12%), respectively. Figure 2 shows photographs of aortas isolated from representative animals of each of the four treatment groups.

Endothelium-Dependent Vascular Responses

Basal EDNO release. Endothelium-dependent increase in isometric tension in response to 100 µM L-NNA (basal EDNO production) of aortic rings contracted with 3 µM PE is shown in Fig. 3. The mean ± SE contractions of the vessels in response to 3 µM PE in the four treatment groups were as follows: VC, 2.95 ± 0.07 g; L-NAME, 3.03 ± 0.08 g; E2, 3.18 ± 0.04 g; L-NAME + E2, 2.96 ± 0.07 g (n = 8 in each group). These values were not statistically different. Basal EDNO-mediated responses were significantly (P < 0.05) diminished in rings isolated from the L-NAME-treated rabbits compared with controls. The responses in the L-NAME-treated group were not statistically different from those obtained in endothelium-denuded segments (data not shown), indicating complete inhibition of basal EDNO production. This observation demonstrates that chronic treatment with L-NAME results in severe inhibition of unstimulated NOS III activity in rabbit thoracic aortas.

Endothelium-intact rings from the E2 group responded with significantly (P < 0.05) greater tension development to L-NNA, indicating significantly increased release of EDNO in these vessels. 17β-Estradiol administration to the L-NAME-treated rabbits resulted in significant increase in basal EDNO release even in the presence of the NOS inhibitor compared with the L-NAME-treated group, although EDNO-mediated responses became significantly (P < 0.05) suppressed compared with the E2 group.

The percent increase in basal EDNO release in response to chronic 17β-estradiol treatment was not statistically different in the absence (85.7 ± 9%) and presence of L-NAME treatment (115 ± 34%).

Stimulated EDNO release. Endothelium-dependent relaxation of thoracic aortic rings to ACh is shown in Fig. 4A. L-NAME treatment resulted in a partial, but significant, reduction of the ACh-induced endothelium-dependent relaxation, providing further evidence for the presence of endothelial dysfunction, i.e., inhibition of EDNO production. Responses to ACh of the aortic rings from the 17β-estradiol-treated animals in the presence and absence of L-NAME administration were not different from the responses of the intact aortic rings from the vehicle-treated control group.

Endothelium-independent relaxation. Endothelium-independent relaxation to NG was not different between aortic rings isolated from animals in the four treatment groups (Fig. 4B).

Serum Total Cholesterol and Estradiol

Cholesterol feeding resulted in a 20-fold increase of total serum cholesterol. There was no significant difference in the serum total cholesterol level between the four different treatment groups (Table 1).

Plasma estradiol was low in the ovariectomized VC group. The L-NAME-treated ovariectomized group had similarly low 17β-estradiol levels as the control group. Daily injection with 30 µg 17β-estradiol resulted in a significant, 10-fold increase in the circulating estrogen level in both the E2 and L-NAME + E2 groups (Table 1).

DISCUSSION

Diminished endothelium-dependent vasodilation has been demonstrated in both clinical (6) and experimen-
tal (5) hypercholesterolemia and atherosclerosis. The impairment in vascular reactivity was largely due to a reduction in EDNO activity and was observed before any detectable morphological changes of atherosclerosis occurred (29). Chronic L-NAME administration to hypercholesterolemic rabbits resulted in significant suppression of basal and stimulated EDNO production with significant acceleration of atherosclerotic plaque development (3, 22), demonstrating the important protective role of EDNO in atherosclerosis. EDNO exerts a wide variety of potentially antiatherogenic effects, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle proliferation, and vasoconstriction (11). Improved endothelial function could potentially contribute to the clinical benefit of patients as has been suggested in the case of lipid-lowering agents (26) and angiotensin-converting enzyme inhibitors (21).

Impaired EDNO availability in atherosclerosis can be the result of multiple mechanisms including increased degradation of NO, reduced availability of substrate or cofactor, presence of endogenous inhibitors, or reduced expression of NOS III (4, 11). The heterogeneity of the cause of endothelial dysfunction may limit the therapeutic strategies used in the treatment of atherosclerosis.

Numerous epidemiological evidence supports the role of estrogens in the reduced incidence of coronary heart disease in hormone replacement-treated postmenopausal women (8, 19, 23). Antiatherosclerotic effect of estrogen involves several mechanisms (8, 19, 23), among which the stimulatory effect on EDNO production has been well documented (1, 7, 10, 12–15, 18, 20, 28). Treatment with estrogens resulted in improved endothelium-mediated vasodilation and NO release (1, 10) or...
increased expression of NOS III, demonstrated both in vivo and in vitro (9, 13, 14, 20, 28).

The role of EDNO in mediating the cardiovascular protection by estrogen has been suggested based on findings that the antiatherosclerotic effect of 17β-estradiol was inhibited by the removal of the endothelium in atherosclerotic rabbits (16) and that decreased accumulation of cholesterol into the aortic wall of hypercholesterolemic rabbits in response to treatment with estrogens was attenuated by L-NAME treatment (15). However, chronic NOS inhibition by L-NAME did not influence the antiatherosclerotic effect of 17β-estradiol in apolipoprotein E-deficient mice (7). This apparent discrepancy between these studies can be explained by differences in the end points measured to document the antiatherosclerotic effect of estrogen, the degree of NOS inhibition achieved, or differences in species. The inhibition of NOS by L-NAME had no significant effect on the measured end points in these studies, indicating that lipid accumulation into the aortic wall (15) or the area of lipid-filled lesions at the level of the aortic valves (7), used to evaluate the extent of atherosclerosis, may not be sensitive enough to detect changes due to EDNO inhibition alone. In addition, none of these studies provided evidence of changes in EDNO-mediated vasorelaxation, as a measure of NOS function in response to L-NAME or 17β-estradiol treatment, in the animals in which the effect on atherosclerosis was investigated.

In our study, plaque-to-surface area ratio of the thoracic aorta and the aortic arch was significantly increased in the L-NAME-treated rabbits that exhibited severe endothelial dysfunction. These results are in agreement with earlier findings of others using plaque-to-surface area ratio for the measurement of atherosclerosis (22). Basal EDNO production, which reflects the level of bioavailable EDNO (assessed by N-nitro-L-arginine (L-NNA; 100 µM)-evoked increases in isometric tension of intact aortic rings contracted with phenylephrine (PE; 3 µM) in organ chambers in presence of 10 µM indomethacin. Data are shown as means ± SE of 8 experiments in each treatment group. Basal EDNO production was significantly (* P < 0.05) inhibited by chronic treatment with L-NAME (open bar) and was significantly (* P < 0.05) enhanced in estrogen-treated group (E2) (large hatched bar) compared with ovariectomized hypercholesterolemic controls (solid bar). Combined treatment with L-NAME and 17β-estradiol (L-NAME + E2) (crosshatched bar) resulted in a significant (+ P < 0.05) improvement of NO-mediated responses compared with L-NAME group.

Fig. 3. Effect of 17β-estradiol and L-NAME treatment on basal endothelium-derived nitric oxide (EDNO) release measured by N-nitro-L-arginine (L-NNA; 100 µM)-evoked increases in isometric tension of intact aortic rings contracted with phenylephrine (PE; 3 µM) in organ chambers in presence of 10 µM indomethacin. Data are shown as means ± SE of 8 experiments in each treatment group. Basal EDNO production was significantly (* P < 0.05) inhibited by chronic treatment with L-NAME (open bar) and was significantly (* P < 0.05) enhanced in estrogen-treated group (E2) (large hatched bar) compared with ovariectomized hypercholesterolemic controls (solid bar). Combined treatment with L-NAME and 17β-estradiol (L-NAME + E2) (crosshatched bar) resulted in a significant (+ P < 0.05) improvement of NO-mediated responses compared with L-NAME group.

Fig. 4. Effect of 17β-estradiol and L-NAME treatment on endothelium-dependent relaxation induced by ACh (10 nM–10 µM) (A) and endothelium-independent relaxation induced by nitroglycerin (1 nM–10 µM) (B) in PE (3 µM)-contracted rings in presence of 10 µM indomethacin. Relaxation is expressed as percent decrease of PE contraction and is shown as mean ± SE of 8 experiments in each treatment group. Open circles illustrate responses of control group (VC). Chronic L-NAME treatment (closed circles) of rabbits significantly (* P < 0.05) attenuated endothelium-dependent responses (A). Open squares show responses of aortic rings isolated from 17β-estradiol-treated rabbits (E2). 17β-Estradiol did not significantly affect endothelium-dependent relaxation in absence (open squares) or presence (solid squares) of L-NAME (A). There was no difference in endothelium-independent relaxation to nitroglycerin among the 4 treatment groups (B).
correlation between the severity of atherosclerosis and the degree of endothelial dysfunction assessed by impairment in endothelium-dependent vasodilation (11).

In the present study, estrogen treatment resulted in significant reduction of atherosclerotic lesion development measured by plaque-to-surface area ratio. The change in lesion area was accompanied by increased basal EDNO-mediated responses, assessed by 

L-NNATreatment resulted in significant increases in EDNO formation by L-NAME. The contribution of the above mechanisms to protection against atherosclerosis may vary depending on the severity of hypercholesterolemia, vascular bed, or species. The involvement of these additional beneficial effects could only be studied under experimental conditions with complete inhibition of EDNO release and prevention of 17β-estradiol-induced increase in EDNO production.

In summary, the present study demonstrates that 17β-estradiol treatment is efficacious in atherosclerosis even in situations when EDNO production is severely impaired. Our results indicate a strong correlation between EDNO production and atherosclerotic lesion development in this animal model of atherosclerosis. The data indicate that, in ovariectomized, hypercholesterolemic rabbits, estrogen-evoked cardiovascular protection is mediated at least in part by increased EDNO production even in the presence of a NOS inhibitor. The fact that the antiatherosclerotic effect of 17β-estradiol persisted after significant impairment of endothelial function by L-NAME suggests that estrogen therapy may exert cardiovascular benefits even in patients with severe endothelial dysfunction, possibly by increasing the expression of NOS III protein.

REFERENCES


H1794 ROLE OF NO IN THE ANTIATHEROSCLEROTIC EFFECT OF ESTROGEN