Physiological Concentration of 17β-Estradiol Retards the Progression of Severe Atherosclerosis Induced by a High-Cholesterol Diet Plus Balloon Catheter Injury

Role of NO

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Abstract—The molecular mechanisms of the antiatherosclerotic effects of estrogen are not yet known. We evaluated the effects of 17β-estradiol (E₂) on high cholesterol diet– (HCD; standard diet and 1% cholesterol) and balloon injury–induced atherosclerosis in female New Zealand White rabbits. The abdominal aortas of 40 oophorectomized (Groups 1 through 5) and 8 nonoophorectomized (Group 6) rabbits were injured by balloon catheter, and the animals were then divided into the following groups and treated for 10 weeks: Group 1, standard diet; Group 2, standard diet plus a moderate dose of E₂ (100 μg · kg⁻¹ · d⁻¹); Group 3, HCD; Group 4, HCD plus a moderate dose of E₂; Group 5, HCD plus a low dose of E₂ (20 μg · kg⁻¹ · d⁻¹); and Group 6, HCD in nonoophorectomized rabbits. After the treatment phase, plasma E₂ was increased up to 282.2 ± 45.5 pg/mL in Group 2, 263.0 ± 41.5 pg/mL in Group 4, 87.9 ± 18.8 pg/mL in Group 5, and 45.6 ± 7.3 pg/mL in Group 6. HCD-mediated increases in plasma lipid levels were not changed by E₂ treatment, whereas E₂ decreased the aortic intimal thickening in Group 2 animals compared with those in Group 1 and reduced atherosclerosis in the thoracic and abdominal aortas of Group 4, 5, and 6 rabbits compared with those in Group 3. E₂ restored the impaired abdominal aortic endothelium–dependent relaxation of balloon-injured and HCD-supplemented rabbits, and E₂ increased basal nitric oxide (NO) release. The basal NO–releasing effect showed a significant, inverse relation with the severity of atherosclerosis. Plasma E₂ concentration also showed a significant, inverse relation with atherosclerotic area. In conclusion, physiological concentrations of E₂ can retard the progression of severe atherosclerosis and stabilize atheromas induced by HCD and balloon injury. The retardation may be partially mediated by endothelial NO function in vessels treated with E₂. (Arterioscler Thromb Vasc Biol 2000;20:1613-1621.)

Key Words: nitric oxide ■ estradiol ■ arteriosclerosis ■ balloon injury ■ endothelium

It is well known that estrogen retards the development of atherosclerosis, but the mechanisms of action are not clearly established. Estrogen replacement therapy suppresses the incidence of cardiovascular disease in postmenopausal women, and it reduces plasma LDL cholesterol and increases HDL cholesterol levels. However, the alterations in lipid profiles reportedly account for only a limited portion of the protective effects of estrogen against cardiovascular disease. Estrogen exerts a direct action on the vessel wall via incompletely understood mechanisms. Recently, estradiol was found to inhibit leukocyte adhesion and transendothelial migration in rabbits, which suggests one of the mechanisms of a direct antiatherosclerotic effect by estrogen on the vessel wall. Nitric oxide (NO), released from endothelial nitric oxide synthase (endothelial NOS), has been demonstrated to offer protection against the development of atherosclerosis by producing vascular dilatation, inhibiting monocyte adhesion to the endothelium, and other modes of action. We have found that estrogen acts via an NO-mediated system in vivo and in vitro. The aortas of intact (nonoophorectomized) female rabbits release a greater amount of NO than do the aortas of oophorectomized female or of male rabbits, and thus, NO might effectively protect against atherosclerosis in female versus male rabbits. 17β-Estradiol (E₂) increases endothelial NOS activity via a receptor-mediated system in cultured endothelial cells. This information led us to investigate the effects of E₂ on atherosclerosis and endothelial dysfunction induced by a high-cholesterol diet (HCD) and balloon injury.

Our preliminary experiment had shown that severe atherosclerosis (almost 75% stenosis) developed in the abdominal aorta in response to cholesterol feeding after balloon injury. We had also shown that an HCD induces atherosclerosis more slowly in female rabbits than in males. The controversy remained as to whether balloon injury diminishes the acetylcholine (ACh)-induced, NO-mediated relaxation and
whether the distance between the endothelium and medial smooth muscle cells inhibits the effects of NO.\textsuperscript{14,15} It is not known whether E\textsubscript{2} can prevent the impairment of endothelium-dependent relaxation or basal NO release in aortas with severe atherosclerosis induced by balloon catheter injury and an atherogenic diet. Therefore, in the current study, we evaluated how E\textsubscript{2} treatment affects basal NO release, as evaluated by contractions in response to N\textsuperscript{0}-monomethyl-L-arginine acetate (L-NMA) and aortic cGMP levels in atherosclerotic vessels. This report shows the antiatherosclerotic effect of E\textsubscript{2} in the aortas of oophorectomized female rabbits treated with an HCD and balloon injury.

\section*{Methods}

\subsection*{Chemicals and Solutions}

ACh, prostaglandin F\textsubscript{2\alpha} (PGF\textsubscript{2\alpha}), calcium ionophore A-23187, indo- methacin, and L-NMA were all purchased from Sigma Chemical Co. Monoclonal antibodies against estrogen receptors-\alpha and -\beta were purchased from Transduction Laboratories. Nitroglycerin (NTG; 10\% wt/wt triturate in lactose) was from Nihon Kayaku Co, Ltd. Krebs-Henseleit solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.4 mmol/L CaCl\textsubscript{2}, 1.2 mmol/L MgSO\textsubscript{4}, 1.2 mmol/L KH\textsubscript{2}PO\textsubscript{4}, 25 mmol/L NaHCO\textsubscript{3}, 11 mmol/L glucose, and 0.002 mmol/L EDTA; pH 7.4) was saturated with 95\% O\textsubscript{2}/5\% CO\textsubscript{2}. The composition of the depolarizing KC\textsubscript{1} solution was similar to that of Krebs-Henseleit buffer, except for the replacement of NaCl by an equimolar amount of KCl. All concentrations shown are those in the final bath.

\subsection*{Animals}

A total of 48 female New Zealand White rabbits, 3 to 4 months old and weighing 2.0 to 2.4 kg, were obtained from Kitayama Rabbits (Ina, Japan). The rabbits were housed individually in stainless steel cages at 20±3\(^\circ\)C with a 12-hour light/dark cycle and with free access to water. All animals were initially fed standard rabbit chow (Oriental Yeast Co, Ltd) for 2 weeks. After 2 weeks of the standard chow diet, 40 rabbits, which were bilaterally oophorectomized, and 8 nonoophorectomized rabbits were used in this study. Six weeks after oophorectomy, the abdominal aortas of all rabbits were injured by balloon as described previously, with slight modification.\textsuperscript{14} In brief, a 3F Fogarty catheter was inserted from the right femoral artery and advanced to a position just below the diaphragm. The ideal luminal area was calculated from the perimeter of the lumen and the internal elastic lamina for the ideal luminal area.

\subsection*{Assays for Tissue Cholesterol Content}

To assay for free and esterified cholesterol content, the 2-cm-long segment of the abdominal aorta, from the portion enclosed by the diaphragm (9 cm distal to the aortic valve) to the portion 11 cm distal to the aortic valve, was weighed, minced, and homogenized in 20 volumes of chloroform/methanol (2:1, vol/vol) containing 0.001% BHT as an antioxidant in a motor-driven, glass-glass homogenizer at 0\(^{\circ}\) to 2\(^{\circ}\)C. Total lipid extraction was carried out in the homogenate by using the Folch procedure. The lipid-containing fraction was dried under N\textsubscript{2} and resuspended in isopropanol. The total cholesterol and free cholesterol levels were measured in the extract by the specific enzymatic assays mentioned in plasma lipid analysis. The esterified cholesterol was calculated as the difference between total and free cholesterol (Wako Pure Chemical Industries, Ltd.).\textsuperscript{20}

\subsection*{Immunocytochemical Analysis}

Cross sections of the descending thoracic aorta, adjacent to each segment taken for evaluation of endothelium-dependent responses, were stained with hematoxylin-eosin to examine the endothelial lining and with van Gieson’s elastic stain to determine the thickness of the intima. Morphometric analysis was performed as described by Weiner et al.\textsuperscript{19} In brief, the complete section in each block was projected onto a vertical surface with a projected microscope and the outlines of the sections were digitized with the objective lens. The contours of the lumen and internal elastic lamina were traced, and the tracings were digitized (PC-9801 ES, NEC) by using a graphics tablet. The mean surface involvement by atherosclerotic lesion per vessel per animal was calculated by summing all results obtained after dividing the lesion circumference by the circumference of the internal elastic lamina and then dividing the sum by the number of sections studied. Circumferences of lesion and normal vessel were defined as circumferences of each part of internal elastic lamina. The area occupied by atherosclerotic lesions was defined as the percent area bounded by the lumen and the internal elastic lamina for the ideal luminal area. The ideal luminal area was calculated from the perimeter of the internal elastic lamina on the assumption that the true shape of the vessel was circular, to exclude the artificial effect due to fixation in 10\% formalin solution. The mean area occupied by the lesions per vessel per animal was calculated by summing the areas occupied by lesion of all sections and dividing the sum by the number of sections per vessel (n=6 for 1 vessel). Data were transferred to a minicomputer (Macintosh Quadra 700, Apple, Ltd) for further analysis.

\subsection*{Determination of Plasma Lipid and E\textsubscript{2} Concentrations}

Total cholesterol and triglyceride levels were measured by enzymatic assays as described previously.\textsuperscript{16,17} The HDL cholesterol was determined after precipitation with phosphotungstate-MgCl\textsubscript{2}.\textsuperscript{17} The plasma concentration of E\textsubscript{2} was quantified as described previously.\textsuperscript{18}

\subsection*{Histological Evaluation of Aortic Atherosclerosis}

After 10 weeks of treatment, the rabbits were killed by exsanguination after being anesthetized with pentobarbital (50 mg/kg IV). Cross sections of the descending thoracic aorta and abdominal aorta, adjacent to each segment taken for evaluation of endothelium-dependent responses, were stained with hematoxylin-eosin to examine the endothelial lining and with van Gieson’s elastic stain to determine the thickness of the intima. Morphometric analysis was performed as described by Weiner et al.\textsuperscript{19} In brief, the complete section in each block was projected onto a vertical surface with a projected microscope and the outlines of the sections were digitized with the objective lens. The contours of the lumen and internal elastic lamina were traced, and the tracings were digitized (PC-9801 ES, NEC) by using a graphics tablet. The mean surface involvement by atherosclerotic lesion per vessel per animal was calculated by summing all results obtained after dividing the lesion circumference by the circumference of the internal elastic lamina and then dividing the sum by the number of sections studied. Circumferences of lesion and normal vessel were defined as circumferences of each part of internal elastic lamina. The area occupied by atherosclerotic lesions was defined as the percent area bounded by the lumen and the internal elastic lamina for the ideal luminal area. The ideal luminal area was calculated from the perimeter of the internal elastic lamina on the assumption that the true shape of the vessel was circular, to exclude the artificial effect due to fixation in 10\% formalin solution. The mean area occupied by the lesions per vessel per animal was calculated by summing the areas occupied by lesion of all sections and dividing the sum by the number of sections per vessel (n=6 for 1 vessel). Data were transferred to a minicomputer (Macintosh Quadra 700, Apple, Ltd) for further analysis.
antibody-positive cells and analyzed statistically as described in a previous report.\(^2\) Five samples were prepared from each rabbit.

### Isometric Tension Measurement

The thoracic and abdominal aortas of the rabbits were removed carefully to protect the endothelial lining, cleared of adhering fat and connective tissue, and cut into 2-mm-wide transverse rings. The thoracic aorta was taken from the orifice of the left first costal artery (~4 cm distal to the aortic valve) to the 3 cm above the portion enclosed by the diaphragm (~7 cm distal to the aortic valve). The abdominal aorta was taken from 4 cm below the portion enclosed by the diaphragm (13 cm distal to the aortic valve) to 3 cm distal to the bifurcation of the internal iliac arteries. The optimal passive load for both control and atherosclerotic aortas was determined as the contractile response to 122 mmol/L KCl. Before starting the experiments, the rings were stretched to their predetermined optimal force, mounted on stainless steel hooks in 20-mL-capacity muscle chambers, and bathed in Krebs-Henseleit solution, pH 7.4 at 37°C for 1 hour. Force was measured isometrically with a force displacement transducer (model DSA-603, Minebea Co, Ltd) and displayed on a multiten recorder (model R-60, Rika Denki Co, Ltd). Experiments were conducted to determine the responsiveness of the endothelium-intact aortic rings to an endothelium-dependent vasodilator, ACh. The responsiveness of the endothelium-denuded aortic rings to the endothelium-independent vasodilator, NTG, was also determined. In these experiments, PGF\(_{2\alpha}\) (2.6×10\(^{-5}\) mol/L) initially induced the submaximal force. To investigate the tone-related release of NO from the endothelium-intact aortic rings, moderate vascular tone (35% to 50% of the contractile response obtained with 122 mmol/L KCl) was induced by low PGF\(_{2\alpha}\) concentrations (0.8×10\(^{-6}\) mol/L). Concentration-related contractile responses to L-NMA (10\(^{-6}\) to 10\(^{-4}\) mol/L) were assessed.\(^9\) In some experiments, indomethacin (5×10\(^{-6}\) mol/L) was added to the muscle chambers for 60 minutes before the start of the experiment to rule out the contribution of prostanoids.

### Measurement of Nitrite and Nitrate (NO\(_2^-\)/NO\(_3^-\))

The plasma concentrations of NO\(_2^-\)/NO\(_3^-\) (NO\(_x\)) were measured with an automated NO detector—high-performance liquid chromatography system (ENO10, Eicom Co) as described previously.\(^2\) In brief, samples were collected in an automated sample injector connected to an automated NO detector. NO\(_x\) and NO\(_3^-\) in the dialysates were separated by a reverse–phase separation column packed with poly-styrene polymer (NO-PAK, 4.6×50 mm; Eicom), and NO\(_3^-\) was reduced to NO\(_x\) in a reduction column packed with copper-plated cadmium (NO-RED, Eicom). NO\(_x\) was mixed with a Griess reagent (0.5% sulfanamide, 0.025% N-(1-naphthylethy)ethylendiamine di-hydrochloride, and 1.25% HCl) to form a purple azo dye in a reaction coil. The color of the product of the Griess reaction was measured at 540 nm by a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 mL/min, was 10% methanol containing 0.15 mol/L NaCl/NH\(_4\)Cl and 0.5g/L disodium EDTA.

### Plasma Biochemical Profile

<table>
<thead>
<tr>
<th>Group</th>
<th>T-Chol, mg/dL</th>
<th>TG, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>E2, pg/mL</th>
<th>cGMP, pmol/g Wet Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>77.8±9.2</td>
<td>46.0±4.2</td>
<td>32.2±4.4</td>
<td>16.4±4.2</td>
<td>8.44±0.71</td>
</tr>
<tr>
<td>Group II</td>
<td>65.2±10.7</td>
<td>37.3±4.3</td>
<td>31.0±1.2</td>
<td>282.2±140.5*</td>
<td>10.85±1.4*</td>
</tr>
<tr>
<td>Group III</td>
<td>1966.5±214.2*</td>
<td>184.3±27.9*</td>
<td>25.8±2.3</td>
<td>11.5±2.7</td>
<td>8.69±0.61</td>
</tr>
<tr>
<td>Group IV</td>
<td>1651.5±124.3*</td>
<td>144.1±27.0*</td>
<td>28.3±3.9</td>
<td>263.0±41.5†</td>
<td>15.2±1.1†</td>
</tr>
<tr>
<td>Group V</td>
<td>1687.7±70.5*</td>
<td>146.8±15.2*</td>
<td>26.4±1.9</td>
<td>87.9±18.8†</td>
<td>12.3±1.5†</td>
</tr>
<tr>
<td>Group VI</td>
<td>1718.9±157.3*</td>
<td>161.9±28.3*</td>
<td>24.9±2.8</td>
<td>45.6±7.3*</td>
<td>11.5±1.6†</td>
</tr>
</tbody>
</table>

*P<0.05 vs Group I; †P<0.05 vs Group 3.

### Determination of cGMP

The cGMP concentration in the homogenates of aortas was determined by a specific radioimmunoassay (RPN226, Amersham).\(^2\) In brief, 4 aortic rings from the segment of abdominal aorta from 2 cm distal to the portion enclosed by the diaphragm (11 cm distal to the aortic valve) to 2 cm distal to the bifurcation of the internal iliac arteries were incubated for 30 minutes in test tubes containing Krebs-Henseleit buffer saturated with 95% O\(_2\)/5% CO\(_2\). The rings were frozen in LN\(_2\), and stored at −80°C. To determine cGMP levels in aortic rings, they were homogenized in 1 mL of 6% trichloroacetic acid at 4°C and centrifuged at 12 000g for 5 minutes. The supernatant was washed 4 times with 4 mL of water-saturated ethyl ether. Liquid samples were then frozen at −80°C and lyophilized overnight. The lyophylate was resolubilized in 1 mL of 0.05 mol/L sodium acetate buffer, and 50-μL aliquots were placed in test tubes. Samples were assayed by a radioimmunoassay for cGMP.\(^2\) Solids left from the initial homogenization step were digested in 1 mL of 0.1N NaOH overnight, after which total protein was determined.

### Data Analysis

Relaxation was measured as the percentage of decrease in force below that evoked by PGF\(_{2\alpha}\) in arterial rings. Contraction in response to L-NMA was measured as the percentage of increase in force above that evoked by PGF\(_{2\alpha}\) in arterial rings. Results were expressed as mean±SEM and represent unpaired data. Data were compared by ANOVA with repeated measures. When a significant F value was found, Scheffe’s test for multiple comparisons was used to identify differences among groups. A level of P<0.05 was considered statistically significant.

### Results

#### Plasma Lipid and E\(_2\) Concentrations

There was no significant difference in serum HDL cholesterol, total serum protein, and body weight in all groups of rabbits over the course of study. In the animals receiving regular chow (Groups 1 and 2), total serum cholesterol or triglycerides did not change significantly over the course of the study. The addition of 1% cholesterol to the diet (Groups 3 through 6) significantly increased total cholesterol and triglyceride levels compared with their respective values in Group 1 animals (the Table). Treatment with E\(_2\) did not affect plasma lipid levels in this study. The plasma concentration of E\(_2\) was 415.5±93.5 and 132.5±23.1 pg/mL, respectively, in Groups 4 and 5 after 4 hours of E\(_2\) administration.

#### Histological Examination of Atherosclerosis

Histological examination of the thoracic aortas revealed more atheromatous lesions, as indicated by the mean percentage of luminal encroachment and mean lesion area, in the hypercholesterolemic (Group 3) group than in the E\(_2\)-treated groups.
The area of atherosclerosis in the thoracic aorta was reduced to 20% by treatment with a moderate dose of E2 in Group 4, and a 70% decrease was observed after low-dose E2 treatment in Group 5 compared with the HCD group (Group 3; Figure 1, left). An 50% decrease was observed in the nonoophorectomized rabbit aortas (Group 6; Figure 1, left). Histologically, the abdominal aortas of rabbits revealed more atherosclerotic lesions than did the thoracic aortas. The abdominal aortas of HCD-fed and balloon-injured rabbits (Group 3) showed severe atherosclerosis with almost 75% stenosis, although this was improved significantly in response to E2 treatment in Groups 4 and 5. E2 treatment in the rabbit group fed a regular diet inhibited the intimal thickening after endothelium denudation by balloon injury (Group 1 versus Group 2). The atherosclerotic area of abdominal aorta was 60% diminished by a moderate dose of E2 in Group 4, by 40% by a low dose of E2 in Group 5, and by 25% in nonoophorectomized rabbits (Group 6) compared with the HCD group (Group 3; Figure 1, right). These results suggest that E2 can retard the progression of complicated atherosclerosis induced by an HCD and balloon injury.

Aortic Cholesterol Content
The total and esterified cholesterol contents in the vessels exhibited the same tendency as the atherosclerotic areas. Free cholesterol levels increased as well in response to cholesterol feeding, although the change was not significant compared with the total and esterified cholesterol content (Please see Figure I online at http://atvb.ahajournals.org).

Immunocytochemical Analysis
Atheroma in the abdominal aorta was composed of many macrophage-derived foam cells and intimal smooth muscle cell proliferation (Figure 2, top). A significant antiatherosclerotic effect of estrogen was shown, and the relative number of macrophages decreased in this study. Estrogen treatment in Groups 4 and 5 not only reduced the area of atherosclerosis but also decreased the area occupied by macrophages (Figure 2, bottom).

Endothelium-Dependent and -Independent Relaxations
In all experimental groups, the endothelium-dependent vasodilator ACh produced concentration-dependent relaxations of the precontracted, thoracic aortic rings with an intact endothelium (Figure 3, left). The magnitude of relaxation of thoracic aortic rings from the hypercholesterolemic animals (Group 3) was diminished. There was no significant difference in endothelium-dependent relaxation observed among the thoracic aortic rings obtained from the normocholesterolemic groups (Groups 1 and 2) or from the hypercholesterolemic animals that had been administered moderate or low doses of E2 (Groups 4 and 5). Endothelium-dependent relaxation in hypercholesterolemic, nonoophorectomized female rabbits (Group 6) showed an intermediate response between HCD rabbits (Group 3) and the treatment group given a low dose of E2 (Group 5).

The endothelium-independent vasodilator NTG produced concentration-dependent relaxations in precontracted, endothelium-denuded thoracic aortic rings. No significant difference in endothelium-independent relaxation was observed in (Group 4 or 5) or the nonoophorectomized group (Group 6).
the aortic rings of all groups of rabbits (Figure 3, middle). The inhibition of NOS by L-NMA (10^{-7} to 10^{-4} mol/L) led to a contractile response in aortic rings precontracted with PGF_2\alpha. The contractile response to L-NMA was concentration dependent, and its magnitude was decreased in endothelium-intact aortic rings of atherosclerotic rabbits (Group 3) compared with rings from rabbits treated with the normal diet (Groups 1 and 2; Figure 3, right). The L-NMA contractile response was higher in E_2-treated rabbits (Groups 4, 5, and 6) than in untreated, atherosclerotic animals (Group 3). Preincubation with indomethacin did not affect the endothelium-dependent relaxation.

Balloon injury and an atherogenic diet diminished the ACh-induced, NO-mediated relaxations in the abdominal aortas of rabbits (Figure 4, left). The ACh-mediated relaxation was improved significantly by E_2 treatment in rabbits fed the regular diet (Group 1 versus Group 2). The ACh-mediated relaxation was significantly abolished in the abdominal aortas of Group 3 rabbits (Figure 4, middle). E_2 dose-dependently restored relaxation in the abdominal aortic rings of Group 4, Group 5, and nonoophorectomized rabbits (Group 6). There was no significant difference observed in endothelium-independent relaxation in all groups of rabbit aortic rings (Figure 4, middle). Inhibition of NOS by L-NMA (10^{-7} to 10^{-4} mol/L) led to a contractile response in abdominal aortic rings precontracted with PGF_2\alpha, which was smaller than that in the thoracic aorta. The L-NMA contractile response was concentration dependent, and its magnitude was decreased in endothelium-intact aortic rings of atherosclerotic rabbits (Group 3) compared with rings from normal diet–treated rabbits (Groups 1 and 2; Figure 4, right). The L-NMA contractile response was higher in E_2-treated rabbits (Groups 4, 5, and 6) than in untreated, atherosclerotic animals (Group 3).

**Plasma Nitrite and Nitrate (NO_2^-/NO_3^-) and Aortic cGMP Concentrations**

The plasma concentration of NO_x (NO_2^-/NO_3^-) was 42.9\pm5.7, 50.2\pm4.2, 48.3\pm4.7, 66.1\pm5.2, 57.9\pm4.1, and 54.6\pm2.9 \mu mol/L, respectively, in Groups 1 through 6. E_2 treatment or no oophorectomy (Groups 2, 4, 5, and 6) tended
to have increased concentrations of NO$_2$/$\text{NO}_3$ in plasma compared with the other 2 groups of rabbits (Groups 1 and 3); however, these differences did not achieve statistical significance ($P<0.061$, Group 3 versus Group 4). Hyperlipidemia did not affect the concentration of NO$_2$/$\text{NO}_3$ in the present study. E$_2$ treatment increased the cGMP level in both the normal and the hypercholesterolemic diet–supplemented rabbits. Thus, cGMP levels were higher in Group 2 than in Group 1 animals and in Group 4 and 5 versus Group 3 animals (the Table). The cGMP level in Group 6 rabbits was between the levels of Groups 3 and 4 (the Table).

Relation of Estrogen Dose to Antiatherosclerotic and NO-Releasing Effects in Rabbit Aortas

We used multiple regression analysis to investigate the relation of plasma estrogen concentration, its antiatherosclerotic effect in rabbit abdominal aortas, and the NO-releasing effect as determined by aortic cGMP concentrations (Figure 5). Plasma estrogen levels showed a dose-dependent antiatherosclerotic and NO-releasing effect, although $R^2$ was smaller than that for the relation between estrogen concentration and atherosclerotic area. Thus, E$_2$ treatment might show an antiatherosclerotic effect in these ways. The physiological and human replacement dose of estrogen might act by increasing basal NO levels, although it is possible that some antiatherosclerotic effects of estrogen may be unrelated to the effects of NO in rabbit atherosclerosis.

**Discussion**

The aim of the present study was to shed light on the effect of E$_2$ on severe atherosclerosis and to investigate its possible mechanism(s) of action on atherosclerosis. Our experiments were specifically designed to evaluate the effects of E$_2$ on the progression of atherosclerosis induced by balloon catheter injury and an atherogenic diet and on the atherosclerosis-associated impairment of endothelial NO function.

Plasma E$_2$ concentrations were achieved by treatment with 2 doses of E$_2$: 1 was a moderate dose (100 $\mu$g · kg$^{-1}$ · d$^{-1}$, yielding a mean E$_2$ concentration of 263.0±41.5 pg/mL in Group 4) and the other was a low dose (20 $\mu$g · kg$^{-1}$ · d$^{-1}$, yielding a mean E$_2$ concentration of 87.9±18.8 pg/mL in Group 5) in HCD-induced atherosclerotic rabbits. These plasma E$_2$ concentrations are almost identical to those of the human menstrual phase and are also seen in patients receiving E$_2$ replacement therapy after menopause. As a control study, we examined female rabbits without oophorectomy (Group 6); their plasma E$_2$ concentration (45.6±7.3 pg/mL) is the true physiological level in rabbits. E$_2$ treatment in oophorectomized and nonoophorectomized rabbits did not produce any significant variations in plasma lipid levels (the Table). However, the present study did show that E$_2$ administration retarded the progression of severe atherosclerosis induced by an HCD plus balloon injury in the rabbit aorta. The mechanisms of the antiatherosclerotic effects of E$_2$ may be due to a direct effect on the vessel wall. In fact, E$_2$ treatment improved
the HCD- and balloon injury–induced atherosclerosis and restored the impaired endothelial function in this study. We analyzed these effects in the thoracic and abdominal aortas.

The objective of our study in the thoracic aorta was to evaluate the effect of estrogen on HCD-induced atherosclerosis. Our results support the finding of an antiatherosclerotic effect of estrogen described previously. However, the current study demonstrated that the antiatherosclerotic effect of E2 is dose dependent. E2 treatment increased NO-mediated vascular responses and basal NO release that had been decreased in atherosclerosis. We speculate from these results that protection of endothelial function, especially of basal NO release, may contribute to the protection against atherosclerosis.

The experiment in abdominal aortas of rabbits fed the HCD after balloon injury was carried out to investigate the effects of estrogen on atherosclerosis and endothelial function recovery after endothelial injury. The pathological findings in abdominal aortas were similar to the findings in restenotic lesions after percutaneous transluminal coronary angioplasty...
in human coronary arteries, especially in plaques prone to rupture, which are rich in macrophage-derived foam cells and contain large amount of lipid.26,27 The findings in our study included intimal smooth muscle cell proliferation and the appearance of some macrophage-derived foam cells. The significant antiatherosclerotic effect of estrogen in this study may be due to the decreased number of macrophages in the atheromas of rabbit aortas after E2 treatment. This might mean that estrogen also plays a role in stabilizing the atheroma; however, the effect of physiological concentrations of E2 on the abdominal aorta was observed to be weak compared with that observed in the thoracic aorta. A significant, inverse relation between basal NO and the severity of atherosclerosis was observed statistically. The expression of inducible NOS and peroxynitrite, a reaction product of NO and O2, has been reported in atherosclerosis.28 However, our study showed that inducible NOS–positive cells were very sparse in the abdominal aortas of rabbits. In addition, areas positive for nitrotyrosine, a marker of peroxynitrite, were not different in the abdominal aortas of Group 3 and 5 rabbits (data not shown). Vascular responses to the endothelium-independent vasodilator NTG were also not different among the 6 groups of animals with HCD-induced atherosclerosis in thoracic aortas and in abdominal aortas subjected to balloon injury plus HCD-induced atherosclerosis. On the other hand, NTG was more potent in the thoracic aortas than in abdominal aortas (Figures 3 and 4). We speculate that severe and encroached whole-vessel atherosclerosis in the abdominal aorta may affect not only the initial preconstriction force but also vessel elasticity in response to NTG-induced relaxation.

Our present results contrast with previous reports describing abolition of the antiatherosclerotic effect of estrogen on severe atherosclerosis induced by an HCD and balloon injury.29,30 The difference between our results and previous reports may be due to the following reasons. We did not clamp the plasma cholesterol level, which may have had some additional effects, although these levels did not show any statistically significant differences. Second, the dose and mode of estrogen treatment were different. We selected 2 doses to achieve moderate (ie, preovulation levels in young women) and physiological (ie, replacement levels in postmenopausal women) concentrations. Then we examined the nonoophorectomized female rabbits to evaluate the effect of a truly physiological concentration of estrogen in rabbits. The plasma E2 concentration obtained by low-dose E2 supplement (Group 5) was higher than that in nonoophorectomized rabbits (Group 6). In other words, the replacement level of plasma E2 for humans was higher than the true E2 physiological concentration for rabbits; thus, a stronger antiatherosclerotic effect was observed in Group 5 than in Group 6. As a result, we could compare the effects of various concentrations of E2, which showed an antiatherosclerotic effect in a dose-dependent manner (Figure 5). Our selection of subcutaneous injection as a route of administration for E2 may have abolished the adverse effect that oral supplementation with estrogen may produce on triglycerides, which is a result of its passage through the liver. Last, the beginning of HCD feeding that occurred 6 weeks after oophorectomy was different from that of previous studies, in which HCD feeding was started immediately after oophorectomy. It has been suggested that an observation period of 4 weeks is necessary to achieve a nonestrous status after oophorectomy and to obtain confirmation of no significant estrogen release.31

It is important to determine the role of NO in relation to the antiatherosclerotic effect of estrogen. NO has antiatherosclerotic effects of its own, and the inhibition of intrinsic NO synthesis by NOS inhibitors is known to worsen atherosclerosis.8 The present study shows that balloon injury and an atherogenic diet (ie, endothelial denudation and hyperlipidemia) significantly diminished the ACh-induced, NO-mediated relaxation in the abdominal aortas of Group 3 rabbits. We also studied how E2 treatment affects basal NO release, as evaluated by contraction to L-NMA and aortic cGMP levels in atherosclerotic vessels. A significant, inverse relation between cGMP concentration and the severity of atherosclerosis was observed statistically. This may also imply that basal NO release plays an important role in the antiatherosclerotic effect of NO. E2 treatment increased basal NO release in a dose-dependent manner in atherosclerotic vessels, and our previous study showed an increased release of NO in cultured bovine and human endothelial cells in response to estrogen.12 We have also shown that basal NO release is greater in female rabbits than in male or oophorectomized female rabbits, and it is possible that a high concentration of E2 in female rabbits significantly retards atherosclerosis progression than in males due to a higher level of basal NO release in females.10,11 Estrogen has also been reported to be effective for neovascularization and endothelial repair in rabbit vessels by means of an NO-dependent mechanism.32,33 The increase in basal NO release by a low dose of extrinsic estrogen in this study may indicate that the antiatherosclerotic effect of estrogen occurs through NO.30,11 Furthermore, this assumption is supported by a recent study, which showed that inhibition of NOS reduced the antiatherosclerotic effect of estrogen in cholesterol diet–induced atherosclerosis in the rabbit aorta.34 However, we must consider another possibility that improved endothelial function due to estrogen treatment was not only the cause of the antiatherosclerotic effect but also the result of diminished atherosclerotic lesion formation through mechanisms other than NO. Some reports have described that the effects of estrogen are not through NO but through their dependence on another system and have concluded that NO does not play a role in some types of atherosclerosis.35,36 Additional study may be necessary to evaluate the antiatherosclerotic mechanisms of E2 on HCD- and balloon injury–induced atherosclerosis.

Regarding the mechanism for the antiatherosclerotic effects of estrogen, we can rule out the role of estrogen receptors on the vessel wall.37 Estrogen receptor-β was recently identified, and we have observed in preliminary studies that its distribution increases in the atherosclerotic rabbit aorta (data not shown). Estrogen has been shown to have a direct vascular effect in estrogen receptor-α–knockout mice, which suggests the important role of estrogen receptor-β. As these results are consistent with ours, it may be important to clarify the mechanism of estrogen’s effect on atherosclerosis with regard to estrogen receptors.38

In conclusion, the restoration of NO release in response to E2 protects endothelial function in atherosclerotic vessels, and physiological concentrations of E2 can retard the progression of severe atherosclerosis and stabilize atheromas induced by an HCD and balloon injury. The present study indicates a
favorable future for estrogen replacement therapy for the treatment of atherosclerosis.

Acknowledgment

This study was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan, No. 05670603.

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Physiological Concentration of 17β-Estradiol Retards the Progression of Severe Atherosclerosis Induced by a High-Cholesterol Diet Plus Balloon Catheter Injury: Role of NO
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Arterioscler Thromb Vasc Biol. 2000;20:1613-1621
doi: 10.1161/01.ATV.20.6.1613
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Figure I (on line only)

Left: The content of total cholesterol (TC) in rabbits' abdominal aortae. Right: The content of esterified cholesterol (EC) in rabbits' abdominal aortae. The lipid contents were measured from six groups of rabbit (Gp.I: standard diet, Gp.II: standard diet plus moderate dose of $17\beta$ estradiol, Gp.III: high cholesterol diet [standard diet plus 1% cholesterol], Gp.IV: high cholesterol diet plus moderated dose of E$_2$, Gp.V: high cholesterol diet plus low dose of E$_2$, Gp.VI: high cholesterol diet on non oophorectomized females). The level of significance was indicated as *$p<0.05$, **$p<0.01$
Plasmatic Concentration of $17\beta$ estradiol (pg/ml)

cGMP (pmol/wet g tissue)