Estrogen Modulates Responses of Atherosclerotic Coronary Arteries

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Although evidence indicates that estrogen replacement therapy reduces risk of coronary heart disease, the mechanism remains unknown. Among the possibilities are that estrogen replacement therapy may 1) inhibit growth of atherosclerotic plaque and 2) decrease the prevalence of transient myocardial ischemia and myocardial infarction by modulating vasomotion in atherosclerotic coronary arteries. Using quantitative coronary angiography, we determined vasomotor responses of atherosclerotic coronary arteries in ovariectomized cynomolgus monkeys; six were given physiological estrogen “replacement” by subcutaneous implants, and six were not. Intracoronary infusion of the endothelium-dependent dilator acetylcholine (1 × 10⁻⁶ M) caused paradoxical constriction of coronary arteries (from 1.2±0.2 to 0.6±0.1 mm, p<0.05) in the estrogen-deficient monkeys. However, acetylcholine tended to minimally dilate the left circumflex coronary artery in estrogen-treated monkeys (from 1.2±0.2 to 1.5±0.2 mm, p>0.2). Although estrogen replacement therapy reduced plaque extent in coronary arteries, altered vasomotion was not related to plaque extent. We conclude that estrogen modulates vasomotion of atherosclerotic coronary arteries of monkeys and speculate that estrogen-modulated constrictor responses of atherosclerotic coronary arteries may reduce the incidence of coronary heart disease in postmenopausal women. (Circulation 1990;81:1680–1687)

Premenopausal white women are at lower risk of coronary heart disease (CHD) than are white men of similar age. Ovarian estrogen is believed to decrease the risk of CHD. It remains unproven, however, whether coronary heart disease risk in women is influenced by conditions that affect endogenous estrogen levels (e.g., menopause). However, estrogen replacement therapy in postmenopausal women is generally agreed to reduce the risk of coronary heart disease relative to the risk in postmenopausal women who do not receive estrogen replacement.

Estrogen replacement therapy may protect against coronary heart disease by altering plasma lipoprotein concentrations (i.e., increasing high density lipoprotein [HDL] cholesterol and decreasing low density lipoprotein [LDL] cholesterol) and thereby inhibiting progression of coronary artery atherosclerosis. Alternatively, estrogen may, through inhibitory effects on arterial vasomotion or thrombosis, protect against clinical events (i.e., transient myocardial ischemia and myocardial infarction).

Endothelial dysfunction in atherosclerotic arteries may result in altered vasomotion that may lead to vasospasm and transient ischemia in the myocardium and cerebral circulation of human beings and nonhuman primates. Whether sex steroids influence these responses has not been determined. The purpose of this study was to examine the effect of estrogen replacement therapy on vasomotor responses of atherosclerotic coronary arteries in ovariectomized monkeys.

Methods

Twelve ovariectomized, adult, female cynomolgus monkeys (Charles River Research Primates, Port Washington, New York) were used in this study. Atherosclerosis was induced by feeding an atherogenic diet for 30 months. Monkeys were fed a diet containing 0.25 mg cholesterol/cal for 16 months. To ensure progression of atherosclerosis, we increased the cholesterol content of the diet to 0.40 mg cholesterol/cal for 8 months and then returned to the original diet (0.25 mg/cal) for an additional 6 months. Six monkeys were treated for the final 26 months with estrogen (17-B estradiol) delivered from a subcutaneous implant. Estrogen concentrations in the blood were determined by the methods of Cronin and Koritnik. Six monkeys (control group) received no
hormonal replacement. Venous blood samples were obtained at 3-month intervals while the monkey was sedated with ketamine HCl 12 mg/kg i.m. Total plasma cholesterol, HDL cholesterol, and triglyceride levels were determined by the methods of the Lipid Research Clinics Program, Department of Health, Education, and Welfare. Measurement of Hormonal Plasma Cholesterol, At Sedated with Light Anesthesia, and the Animals were Doses of Light Anesthesia, and the Animals were Periodic doses of both agents were given to maintain light anesthesia, and the animals were allowed to breathe spontaneously.

A catheter was inserted into the right femoral artery and advanced to the midthoracic aorta for measurement of blood pressure and heart rate. A custom-designed 3F, tapered to 1.8F, catheter (Cook, Inc.) was inserted into the left femoral artery and advanced under fluoroscopic guidance into the left main coronary artery. Blood pressure was monitored from the tip of the coronary catheter to make sure placement of the catheter did not decrease blood pressure in the coronary artery.

Dilator responses in coronary arteries were evaluated by measuring coronary artery diameter with quantitative angiography. Coronary artery diameter measurements were taken from single-plane (ventrodorsal) cut film taken at the midpoint of a 2-ml injection of nonionic contrast medium (Omnipaque, Squibb, Princeton, New Jersey). The 2-ml injection of contrast medium required approximately 2 seconds. The catheter, which was 1 mm in diameter, was used as a size standard to quantify measurements of coronary artery diameter.

In all monkeys, the proximal left circumflex coronary artery was used to measure diameters because it filled with contrast medium more completely and consistently than the left anterior descending coronary artery. The investigator performing angiography and measuring the coronary artery diameters was unaware of which monkey was being evaluated. Coronary artery diameter was measured with calipers at five locations (2 mm apart) along the proximal 1 cm of the circumflex coronary artery. The mean of these measurements was used as the coronary artery diameter for that intervention.

Validation Studies

The technique used to measure coronary artery diameter was validated by identifying objects of known dimensional size. Eight wire rods between 0.5 and 4.0 mm in diameter were radiographed. In addition, holes (0.63–3.98 mm) were drilled into latex plastic blocks and filled with 100% Omnipaque, and the blocks were radiographed next to the coronary catheter (filled with 50% Omnipaque). Diameters of wires, blocks, and catheter were measured with calipers. Calculated diameters were compared with actual diameters. The correlation coefficient and standard error of the estimate were determined between calculated and actual diameters.

For eight metal rods between 0.5 and 4.0 mm in diameter, the correlation coefficient (calculated from eight measurements) between calculated and actual diameters was 0.99 with a standard error of the estimate of 0.05 mm. The variation in the calculated dimensions was ±2%. For cylindrical holes (0.63–3.98 mm in diameter) and the catheter that was filled with 50% contrast medium, the correlation coefficient (calculated from eight measurements) was 0.99, and the standard error of the estimate was 0.1 mm, with 4.0% variability. Therefore, the sensitivity of this method was sufficient to measure changes of approximately 5% in the diameter (1.0–1.5 mm) of coronary arteries of female monkeys. Three measurements were repeated on the same artery. Intraobserver variability was less than 5%.

Interventions

Coronary artery diameter was measured after 2-minute intracoronary infusions of normal saline or acetylcholine (2×10⁻⁵ M/min) and during infusion of nitroglycerin (4 μg/min). The infusion rate for all interventions was 0.25 ml/min. Dosages of acetylcholine and nitroglycerin were based on methods by Ludmer et al. Blood flow to the left ventricle of a 4-kg cynomolgus monkey is approximately 0.8–1.2 ml/min/g. The left ventricle of a 4-kg monkey weighs approximately 10 g. Therefore, total blood flow to the left ventricle is approximately 8–12 ml/min. The left circumflex coronary artery supplies one half of the blood flow to the left ventricle. Therefore, blood flow through the left circumflex coronary artery is approximately 10 ml/min. Thus, the final concentration of acetylcholine in the left circumflex coronary artery was approximately 1×10⁻⁶ M, which was the maximal dose used by Ludmer et al. Blood flow calculations were also used to determine the dose of nitroglycerin.

Measurement of Coronary Artery Atherosclerosis

At the end of the experiment, monkeys were killed with sodium pentobarbital (80 mg/kg i.v.). The cardiovascular system was flushed with normal saline and perfused with 10% neutral buffered formalin at a pressure of 100 mm Hg for 1 hour. The hearts were immersed in 10% neutral buffered formalin. Five serial tissue blocks were cut at approximately 3-mm intervals and perpendicular to the long axis of the left anterior descending coronary artery. Histological sections were stained with Verheoef–van Gieson's stain. These sections were projected, and the cross-sectional area of plaque lesion was measured with a digitizer. Atherosclerosis extent was expressed as the mean cross-sectional area of the intima in millimeters squared.

Values were expressed as mean±SEM. Values in ovariectomized monkeys with and without estrogen replacement therapy were compared by use of the Student's unpaired t test.
FIGURE 1. Histological section of left circumflex coronary artery from an estrogen-treated (left) and an estrogen-deficient monkey (right). Intimal area is 0.34 mm² in the estrogen-treated and 0.24 mm² in the estrogen-deficient monkey; arrows point to internal elastic laminae. Verhoeff-van Giesen’s stain. Original magnification, ×24.

Results

Estrogen Concentrations

Plasma estradiol concentrations in estrogen-treated monkeys were 243 ±30 pg/ml. Estradiol in the plasma of untreated monkeys was below the limits of detection of the assay (<20 pg/ml).

Plasma lipids

Total plasma cholesterol concentrations in estrogen-treated and estrogen-deficient monkeys were 359±45 and 535±74 mg/dl, respectively (p<0.05). HDL cholesterol concentrations in estrogen-treated and estrogen-deficient monkeys were 34±5 and 29±3 mg/dl, respectively (p<0.05). Estrogen replacement therapy resulted in a lower, or less atherogenic, total plasma/HDL cholesterol ratio than in estrogen-deficient monkeys.

Morphology and Morphometry

There was moderate intimal thickening of the left circumflex coronary artery in both groups of monkeys. Monkeys with estrogen replacement had a smaller mean coronary artery intimal area (0.24±0.04 mm²) than monkeys without estrogen replacement (0.37±0.07 mm²) (p<0.05). Histological sections of representative left circumflex coronary arteries are shown in Figure 1.

Cardiovascular Parameters

Mean systemic blood pressure in estrogen-treated monkeys was 99±5 mm Hg during intracoronary infusion of normal saline. Mean blood pressure decreased to 74±9 during infusion of acetylcholine (p<0.05 vs. saline) and to 85±8 mm Hg during infusion of nitroglycerin (p>0.05 vs. saline). Mean systemic blood pressure in estrogen-deficient monkeys was 110±6 mm Hg during intracoronary infusion of saline. Mean blood pressure decreased to 73±5 during acetylcholine (p<0.05 vs. saline) and to 80±7 mm Hg during nitroglycerin infusion (p>0.05 vs. saline). Although infusion of acetylcholine and nitroglycerin systemically affected blood pressure, the changes in blood pressure were similar between groups of monkeys.

Heart rate in estrogen-treated monkeys was 155±35 during saline, 143±20 during acetylcholine, and 150±33 beats/min during nitroglycerin infusion (p>0.05 vs. saline). Heart rate in estrogen-deficient monkeys was 165±25 during saline, 145±30 during acetylcholine (p>0.05), and 163±15 beats/min during nitroglycerin infusion (p>0.05 vs. saline). Therefore, infusion of acetylcholine had a minor effect on heart rate in both groups. Infusion of nitroglycerin had a minimal effect on heart rate in estrogen-treated monkeys but a significant effect on heart rate in estrogen-deficient monkeys.
Effect of Estrogen on Vascular Responsiveness

Coronary artery diameter in estrogen-treated monkeys during saline infusion was 1.2±0.2 mm. The response to infusion of nitroglycerin ranged from moderate dilation to no change in coronary artery diameter (Figure 2, left). The mean coronary artery diameter during the infusion was 1.5±0.4 mm. The responses to infusion of acetylcholine ranged from slight dilation to slight constriction. The mean coronary artery diameter during the infusion was 1.4±0.3 mm (Figure 2, left).

Coronary artery diameter in estrogen-deficient monkeys during saline infusion was 1.3±0.3 mm. The responses to infusion of nitroglycerin ranged from moderate dilation to no change in coronary artery diameter (Figure 2, right). The mean coronary artery diameter during the infusion was 1.4±0.3 mm. Infusion of acetylcholine resulted in a significant decrease in coronary artery diameter in all estrogen-deficient monkeys (Figure 2, right) (*p<0.05 vs. saline control). The mean coronary artery diameter of estrogen-deficient monkeys during the infusion was 0.6±0.2 mm.

Figure 3 is a representative coronary angiogram of an estrogen-deficient monkey, taken during intracoronary infusion of normal saline. This angiogram was taken during systole. Figure 4 is a coronary angiogram taken during infusion of acetylcholine in the same animal. This angiogram was taken during diastole. Pronounced constriction of the proximal left circumflex coronary artery (arrow) was present during infusion of acetylcholine. In four estrogen-deficient monkeys, constriction of coronary arteries in response to acetylcholine was segmental. In two monkeys, constriction involved the entire proximal two thirds of the left anterior descending coronary artery. In all monkeys, the diameter measurement used in calculations was the mean response of the proximal 1 cm of the left circumflex coronary artery. None of the monkeys in either group had occlusive coronary artery disease. According to angiography, the lumens of coronary arteries were not irregular.

To determine whether vascular responsiveness is related to plaque extent, we compared the vascular response to acetylcholine in three pairs of cohort monkeys (estrogen treated and estrogen deficient) that had practically identical plaque extent (Figure 5). In all three pairs, infusion of acetylcholine caused dilation of coronary arteries in estrogen-treated monkeys and constriction in estrogen-deficient monkeys. Therefore, plaque size does not explain differences in vascular responsiveness between estrogen-treated and estrogen-deficient monkeys.

Discussion

The two major findings of this study are that coronary arteries in ovariectomized monkeys had altered vascular responsiveness (paradoxical constriction) to the endothelium-dependent dilator acetylcholine and that estrogen modulated the constrictor response to acetylcholine.

Atherosclerosis impaired endothelium-mediated dilation in coronary arteries of female monkeys. This finding is consistent with other studies that have shown that atherosclerosis impairs endothelium-mediated vascular responses in coronary arteries of male humans,3 and at several arterial sites in male cynomolgus monkeys.4,5,8,9

Our second finding was that estrogen modulated the constrictor responses to acetylcholine. Our finding is consistent with the work of Miller et al10 who reported that estrogen treatment enhanced endothelium-dependent relaxation to acetylcholine in femoral arteries of rabbits. Our study differs in several ways. This is the first report of estrogen’s effect on dilator responses in atherosclerotic coronary arteries in vivo. Also, this study was performed in female monkeys. This is important because coronary artery atherosclerosis in female cynomolgus monkeys is morphologically similar to that of women.11 Female cynomolgus monkeys have a 28-day menstrual cycle and develop diet-induced coronary artery atherosclerosis that is much less extensive than that in male monkeys.11 Also, dyslipoproteinemia and anatomic distribution of plaques in monkeys with diet-induced atherosclerosis is similar to that of women.11

Effects of Estrogen on Plaque Extent

Estrogen replacement therapy decreased plaque extent in coronary arteries. A cohort of estrogen-treated and estrogen-deficient monkeys, with practi-
cally identical plaque extent, were compared with regard to the response of their coronary arteries to endothelium-mediated dilation. Results suggest that estrogen does not alter endothelium-mediated dilation by altering plaque extent (Figure 5).

**Plasma Lipids**

Estrogen replacement therapy decreased total plasma cholesterol and increased HDL cholesterol concentrations. Whether cholesterol concentrations alter endothelium-mediated dilation and constriction of arteries is unclear. Hypercholesterolemia does not augment endothelium-mediated constriction in the hind limb of monkeys.9 However, cholesterol may affect endothelium-mediated dilation in vitro and in vivo.12,13 Although we did not test the following hypothesis, estrogen replacement therapy may alter endothelium-mediated dilation by altering the plasma lipid profile.

**Mechanistic Considerations**

In estrogen-deficient monkeys, infusion of acetylcholine caused constriction of atherosclerotic coronary arteries. This paradoxical constriction in response to acetylcholine has been reported in atherosclerotic coronary arteries of humans.3 Because estrogen did not seem to alter responses to nitroglycerin (Figure 2) in this or another study10 or alter responses to the calcium ionophore A23187,10 estrogen probably does not alter the sensitivity of vascular smooth muscle. However, estrogen may possibly protect against endothelial damage or dysfunction. There are several potential pathways of estrogen action on the endothelium. The effect of estrogen on...
FIGURE 4. Coronary arteriogram showing proximal coronary artery constriction (arrow) in response to intracoronary infusion of acetylcholine in the same animal as in Figure 3. Arteriogram was taken during diastole. Figures 3 and 4 are at the same magnification.

FIGURE 5. Bar graph of percent change in coronary artery diameter from control in three estrogen-treated (OVX+EST) and three estrogen-deficient monkeys (OVX) that were matched for plaque size. Intimal area (IA) (mm$^2$) was used to quantify plaque size and is shown beneath the vascular responses. Infusion of acetylcholine produced different responses in coronary artery diameter between estrogen-treated and estrogen-deficient monkeys with similar plaque size.
endothelium-mediated vascular responses in vitro is not prevented by indomethacin; therefore, prostanoids are probably not involved. Estrogen may increase the release of endothelium-derived relaxant factor. Endothelial cells have been reported to produce a constrictor factor(s). Current candidates for these constrictor factors include thromboxane A2, superoxide anions, and the peptide endothelin. Estrogen treatment may modulate release of a constrictor factor from the endothelium by ADP.

Although we did not test this hypothesis, we speculate that atherosclerosis inhibits release of endothelium-derived relaxant factor, as suggested by Harrison et al. or stimulates release of constrictor factors. Estrogen may facilitate release or response to endothelium-derived relaxant factor and inhibit release or response to constrictor factors.

Whether estrogen acts through a receptor is unclear. Estrogen and progesterone receptors have been found in arterial endothelial and smooth muscle cells of several mammalian species and progesterone receptors have been identified in coronary arteries of humans. A recent study showed that treatment of ovariectomized baboons with 17-B estradiol results in redistribution of aortic intracellular estrogen receptors from the cytoplasmic fraction to the nuclear fraction and an increase in cytoplasmic concentration of progesterone receptors. These findings imply a role for sex steroids in the regulation of arterial cell function. Other animal studies have shown that estrogen treatment results in reductions in lipoprotein-induced aortic smooth muscle proliferation, inhibition of intimal proliferation associated with mechanical endothelial injury, reduced cholesterol ester influx and hydrolysis, inhibition of platelet aggregation, and increased prostacyclin production. These findings provide further evidence that vascular estrogen receptors are physiologically functional and that elevations in circulating concentrations of endogenous estrogen may influence the function of vascular endothelial or smooth muscle cells. Such an effect on endothelial cells could explain the findings reported here.

Methods for Determining Vascular Responses

We used single-plane (ventrodorsal) cut film for angiograms. Although sensitivity may be a little lower with this method than with cineangiography, specificity of response should not be affected. Individual differences in responses to nitroglycerin and to acetylcholine may be due, in part, to the less-sensitive methods used in this experiment (Figure 3). However, this cannot explain the dramatic difference in response to acetylcholine between groups. Infusion of acetylcholine and of nitroglycerin resulted in similar effects on blood pressure and heart rate in both groups. Therefore, altered vascular responsiveness in estrogen-deficient monkeys cannot be explained by passive collapse or reflex stimuli. Measurements of coronary artery diameter, taken at slightly different points of the cardiac cycle, may differ slightly; it is unlikely that these differences can explain the large (50%) decrease in coronary artery diameter of estrogen-deficient monkeys given acetylcholine (Figure 2).

Conclusion

Results of this study indicate that estrogen modulates impaired endothelium-mediated dilation of atherosclerotic coronary arteries. These findings suggest a mechanism by which estrogen replacement may protect against the effects of coronary heart disease in postmenopausal women.

Acknowledgments

We acknowledge the expert technical help of James Roberts, BS, BE, and thank Drs. Carolyn Moyer and Jay R. Kaplan for critical review of this manuscript, Donald R. Koritnik for estrogen assays, and Karen Holbrook for typing this manuscript.

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KEY WORDS: menopause, post, vasospasm, transient ischemia, endothelium, estrogen, atherosclerosis, coronary arteries
Estrogen modulates responses of atherosclerotic coronary arteries.
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Circulation. 1990;81:1680-1687
doi: 10.1161/01.CIR.81.5.1680

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