The Direct Antiatherogenic Effect of Estrogen Is Present, Absent, or Reversed, Depending on the State of the Arterial Endothelium

A Time Course Study in Cholesterol-Clamped Rabbits

Pernille Holm, MD; Heidi L. Andersen, MS, PhD; Malene R. Andersen, MS; Elisabeth Erhardtsen, DVM; Steen Stender, MD, PhD

Background—This study further investigated the relationship between estrogen, arterial endothelium, and nitric oxide (NO) in cholesterol-clamped rabbits.

Methods and Results—Rabbits were ovariectomized, balloon-injured in the thoracic aorta, and grouped to receive cholesterol-enriched chow together with either 17β-estradiol or vehicle for 1, 2, 4, or 8 weeks. In the undamaged aorta, cholesterol accumulation of the placebo rabbits was significantly increased from week 4 to 8 (P<0.001). This increase was almost completely inhibited by estrogen (P<0.001). In the balloon-injured aorta, the estrogen and placebo rabbits accumulated similar amounts of cholesterol in the reendothelialized areas. In the deendothelialized areas, the estrogen group surprisingly accumulated significantly more cholesterol than the placebo group. This difference was apparent from week 2 and became significant at week 8 (P<0.01). Circulating nitrite/nitrate were significantly increased by estrogen at weeks 1, 2, and 4 but not at week 8. Similarly, in additional experiments, basal NO release was significantly higher in estrogen-treated than in placebo-treated rabbits after 4 (P<0.05) but not after 8 weeks. Stimulated NO release and endothelial NO synthase activity did not differ between groups. Mononuclear-endothelial cell binding was reduced by 50% by estrogen after 4 weeks (P<0.05). This difference, however, was abolished by coadministration of NG-nitro-L-arginine methyl ester, an inhibitor of NO production.

Conclusions—The direct antiatherogenic effect of estrogen was present, absent, or reversed, depending on the state of the arterial endothelium, and preceded by a transient increase in NO production followed by a reduced mononuclear-endothelial cell binding. (Circulation. 1999;100:1727-1733.)

Key Words: atherosclerosis ■ balloon ■ endothelium ■ estrogen ■ nitric oxide

Atherosclerosis and its complications remain the most common cause of death in postmenopausal women of the West.1 Several epidemiological studies suggest that estrogen replacement therapy significantly attenuates the progression of this disease.1,2 Underlying mechanisms are not well understood but are thought to involve both effects of estrogen on circulating lipids and lipoproteins and, more importantly, direct effects of estrogen on cells/structures within the arterial wall.

A variety of animal studies support the suggestions of epidemiological studies through the demonstration of a significant inhibition of diet-induced or spontaneous atherosclerosis after estrogen treatment.3-5 However, in these experiments, studies of the direct antiatherogenic effect of estrogen are often hampered by differences in plasma cholesterol levels between groups. We have developed a special model for use of studies of the direct antiatherogenic effect of estrogen: the cholesterol-clamped rabbit. In this model the amount of cholesterol added to the chow of each rabbit is not constant but continuously adjusted according to weekly plasma cholesterol determinations. In this way, all rabbits are maintained at a similar plasma cholesterol concentration, resulting in all aortas being exposed to a similar average plasma cholesterol level. With this model, we have previously shown that the direct antiatherogenic effect of estrogen is abolished by balloon catheter injury,6-8 and significantly attenuated by long-term treatment with NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase (eNOS).7 These findings suggest that the endothelium and endothelial NO are involved in the mechanism by which estrogen inhibits atherogenesis independently of changes in plasma lipids. In the previous experiments, how-
ever, the animals were euthanized 12 weeks after injury. At that time, the balloon-injured area had accumulated much more cholesterol than the surrounding undamaged aorta. Thus, the abolishment of the direct antiatherogenic effect of estrogen by balloon catheter injury could be due to the high accumulation of cholesterol rather than to the state of the arterial endothelium. Furthermore, because the balloon-injured area was not separated into reendothelialized and denuded endothelium, the above experiments did not discriminate between effects of estrogen on aorta with regenerating endothelium and effects of estrogen on aorta where the endothelium is completely absent.

The purpose of the current study was to further investigate the relationship between estrogen, arterial endothelium, and NO in cholesterol-clamped ovariectomized rabbits. In the main experiment (atherosclerosis experiment), the direct effect of estrogen on undamaged and balloon-injured aorta and on circulating plasma nitrite/nitrate levels was studied at different time points during initiation of atherosclerosis (1 to 8 weeks). Before euthanasia, the rabbits were injected with Evans blue dye, enabling separation of the balloon-injured area into reendothelialized tissue and still denuded endothelialized tissue. In 2 additional experiments (NO experiments), the direct effect of estrogen on (I) eNOS activity and basal and stimulated release of NO from the endothelium, and (II) mononuclear-endothelial cell binding in the presence and absence of L-NAME, was studied after 4 (1+II) and 8 (I) weeks of cholesterol feeding.

**Methods**

Sexually mature female New Zealand White rabbits (n=254) were purchased from Interfauna (Huntingdon, England). The rabbits were housed in individual stainless steel cages at a room temperature of 18±2°C with a 12-hour light cycle and free access to drinking water. All experimental procedures were performed in accordance with the Danish regulations for experiments with animals.

**Atherosclerosis Experiment**

**Experimental Design**

One hundred fifty rabbits were anesthetized with intravenous pentobarbital and underwent bilateral ovariectomy and balloon catheter injury in the lower thoracic aorta as described previously.7 8 The rabbits were allowed to recover for 3 weeks before treatment was initiated.

The rabbits were then divided into 10 groups with similar baseline values of plasma cholesterol and body weight to receive intervention with (1) cholesterol-enriched chow together with either 17β-estradiol (17β-E2) cypionate (Sigma) or vehicle (corn oil; Nomeco) for 1, 2, 4, or 8 weeks (estrogen and placebo groups; n=15) or (2) regular (vehicle-enriched) chow together with vehicle for 0 (start of cholesterol feeding period) or 8 weeks (chow groups; n=15). 17 β-E2 cypionate was given subcutaneously in a concentration of 50 μg/kg every 3rd day; vehicle was administered similarly. This dose of estrogen was chosen because it produces plasma estradiol levels in rabbits comparable to those seen in women receiving estrogen replacement therapy.9 Dietary cholesterol was given in individual amounts, aiming at a plasma concentration of about 25 mmol/L in all rabbits.6–8 Blood samples for plasma cholesterol determination were drawn 1 to 2 times weekly and at euthanasia. The final blood samples were additionally used for measurement of circulating nitrite/nitrate levels.

The animals were injected intravenously with 5 mL of Evans blue dye (5 mg/mL), which was allowed to circulate for 5 minutes before the rabbits were killed with an overdose of intravenous pentobarbital.6–9 The aorta was then removed from the level of the aortic valves to the level of the diaphragm, opened longitudinally, and divided into 4 parts: arch (valves to ductus arteriosus; undamaged), upper thoracic (ductus arteriosus to second intercostal arteries; undamaged), lower thoracic (fifth intercostal arteries to celiac artery; balloon-injured), and abdominal (celiac artery to renal arteries; undamaged) aorta. The balloon-injured lower thoracic aorta was further separated into white tissue, consisting of reendothelialized endothelium, and blue tissue, consisting of still denuded endothelium. Each aortic part was fixed with pins on a cork board, and the intima/inner media was stripped from the outer media, weighed, and stored at −20°C until analyzed for aortic cholesterol content.6–8

**Measurement of Circulating Nitrite/Nitrate Levels**

Plasma nitrite/nitrate levels were measured with the use of Griess reagent.10 To reduce the lipid content, plasma samples were initially centrifuged at 14,000 rpm for 2 minutes, and the infranate was carefully removed. This procedure was repeated once. The samples (50 μL) were incubated in microtiter plate wells for 45 minutes at room temperature with 10 μL of 30 μM NADPH and 40 μL of freshly prepared Master Mix (glucose-6-phosphate 15 mmol/L, G6P dehydrogenase 4800 U/L, nitrate reductase 2400 U/L, and NaPi buffer 0.448 mol/L, PH 7.4) to convert nitrate to nitrite. Standards of nitrite and nitrate (5 to 200 μL) were run in parallel. Total nitrite (nitrite+nitrate) was analyzed by reacting the samples with Griess reagent.10
reagent (0.1% N-(1-naphthyl) ethylenediamine HCl and 1% sulfanilamide in 5% phosphoric acid). The absorbance of the reacted samples was read at 540 nm after 10 minutes of incubation at 20°C. The nitrate standards gave absorbance values that were >90% of the corresponding nitrite standards. The interassay variation coefficient for the standards with the same absorbance value as the plasma samples was <2%.

### NO Experiment I

Forty-four rabbits were ovariectomized, individually cholesterol-fed, and treated with either 17β-E2 cypionate or vehicle as described above for 4 or 8 weeks (n=11). At euthanasia, the thoracic aorta was removed and carefully dissected free of connective tissue, for measurement of basal and stimulated NO release, eNOS activity, and aortic cholesterol content (lower 3 cm).6–8

### Basal and Stimulated NO Release

Rings from the upper thoracic aorta were suspended in organ baths for the measurement of isometric tension as described previously.11 In one ring, basal release of NO was determined indirectly by the ratio of contraction evoked by 100 μM of L-NAME (Sigma). In another ring, a cumulative dose-response curve for acetylcholine (Sigma) was performed. Both rings were precontracted with phenylephrine to 30% (basal release) and 50% (acetylcholine-mediated relaxation) of the contractile response evoked by 122 mmol/L potassium. The degree of contractions/relaxations is expressed as a percent of this contraction.

### eNOS Activity

The middle thoracic aorta was removed and transferred to ice-cold, oxygenated saline. The aorta was opened longitudinally, and endothelial cells were obtained by a single scrape with a razor blade along the luminal surface. The cells were placed in Eppendorf tubes containing 50 μL of 50 mmol/L TRIS buffer (pH 7.4) and immediately frozen in liquid nitrogen. The activity of eNOS was determined by conversion of [14C]-l-arginine to [14C]-l-citrulline by a modification of methods previously described.12 In short, the cells were homogenized by 5 cycles of freeze-thawing and incubated for 30 minutes at 37°C in a reaction buffer containing [14C]-l-arginine and calmodulin, tetrahydrobiopterin, FAD, and β-NADPH. [14C]-l-citrulline was isolated by column chromatography and quantified by liquid scintillation counting. To express the activity per endothelial cell, the number of cells in each sample was determined by cell counting in a counting chamber at the light microscopic level. Samples with endothelial cells from pig aorta were used as control material. The interassay variation coefficient was 17%.

### NO Experiment II

Sixty rabbits were ovariectomized, individually cholesterol-fed, and treated with 17β-E2 or vehicle as described above, either alone or together with 160 μg/mL L-NAME in their drinking water for 4 weeks (n=15). At euthanasia, the thoracic aorta was removed and carefully dissected free of connective tissue, for measurement of mononuclear-endothelial cell binding and aortic cholesterol content (lower 3 cm).6–8

### Mononuclear Cell Adhesion Experiment

Mononuclear cell adhesion was determined as previously described.13 Briefly, a 3-cm segment of the upper thoracic aorta was opened longitudinally and, with the endothelial side up, was incubated in a 35-mm culture dish with Hanks’ balanced salt solution (HBSS) on a rocking platform. After 10 minutes, the HBSS medium was replaced by binding medium containing fluorescently labeled human mononuclear cells (5x10⁶ cells/mL). The aortic segment was incubated with the mononuclear cells for 30 minutes, after which the medium was aspirated and the segment washed twice with fresh binding medium. Adherent cells were counted blindly under fluorescence microscopy from 9 predetermined, equally distributed sites.

### Results

#### Atherosclerosis Experiment

**Characteristics of the 10 Rabbit Groups**

The rabbits were well matched with regard to initial levels of body weight and plasma cholesterol (Table). None of the rabbit groups lost weight during the experimental period. The plasma cholesterol level of the chow rabbits was not significantly different at weeks 0 and 8. The cholesterol-fed groups showed a considerable rise in plasma cholesterol levels compared with the chow groups; the mean concentration of plasma cholesterol during the experimental period (AUC/number of days) increased with time (P<0.001 by 2-way ANOVA), but was without difference between the estrogen and placebo group at each time point. The amount of dietary cholesterol, which was used to adjust each rabbit’s plasma cholesterol to 25 mmol/L, increased with time, but was already 2 times higher in the estrogen than in the placebo group after the first week of cholesterol feeding (P<0.001 by 2-way ANOVA).

#### Aortic Atherosclerosis

The data for the undamaged aorta are shown in Figure 1. Aortic cholesterol accumulation of the chow rabbits did not change from week 0 to week 8. Aortic cholesterol accumulation of the cholesterol-fed groups was at a similar low and constant level for the first 4 weeks. From weeks 4 to 8, however, accumulation more than doubled in the placebo group (P<0.001 by multiple comparison). Estrogen almost completely inhibited this increase, resulting in a time-dependent overall effect of estrogen treatment on aortic cholesterol accumulation (P<0.001 by 2-way ANOVA).

The data for the balloon-injured aorta are shown in Figure 2. Cholesterol accumulation of the chow rabbits was similar at weeks 0 and 8 and not significantly different from that in the undamaged aorta in either white (top) or blue (bottom) tissue. Cholesterol accumulation of the cholesterol-fed groups...
increased steadily during the experiment (P<0.001 by 2-way ANOVA) and was higher at all time points than that in the undamaged aorta in both white and blue tissue. The white tissue with regenerated endothelium displayed similar aortic cholesterol accumulation in the estrogen and placebo group at all time points. The blue tissue denuded of endothelium, however, displayed an overall significantly higher aortic cholesterol accumulation in the estrogen than in the placebo group (effect of estrogen treatment, P=0.004 by 2-way ANOVA). This difference was present from week 2, but did not become significant until week 8 (P<0.01 by multiple comparison). The contribution of blue tissue to the total balloon-injured area varied from 30% to 50% (wet weight/wet weight) at the different points and was not significantly different between the estrogen and placebo group.

Circulating Plasma Nitrite/Nitrate Levels
In the chow groups, plasma nitrite/nitrate levels were lower than in the cholesterol-fed groups and showed significantly higher values at week 0 than at week 8 (P=0.01) (Figure 3). In the cholesterol-fed groups, plasma nitrite/nitrate were also significantly affected by time (P=0.02 by 2-way ANOVA); showing an initial increase from week 1 to 2, and a subsequent fall from week 2 to 8. Plasma nitrite/nitrate was markedly increased by estrogen treatment (P<0.001 by 2-way ANOVA). This effect reached statistical significance at week 1, 2 (P<0.05), and 4 (P<0.001) but not at week 8 (Student’s unpaired t test).

NO Experiment I
Three rabbits in the 4-week estrogen group were excluded due to anorexia or resistance to diet-induced hypercholesterolemia. That left 41 rabbits in the experiment. The mean concentration of plasma cholesterol during the experimental period was significantly higher at week 8 than at week 4, but not significantly different between the estrogen and placebo group at each time point (data not shown). As in the above experiment, aortic cholesterol was not significantly different between the 2 groups at week 4 but was significantly lower in the estrogen group than in the placebo group at week 8 (P<0.001 by multiple comparison) (Figure 4, top).
eNOS Activity and Basal and Stimulated NO Release

eNOS activity was similar at week 4 and 8, and not significantly different between the estrogen and placebo group (Figure 4, middle). The magnitude of vasoconstriction induced by L-NAME (basal NO release) was not significantly different at week 4 and 8 (Figure 4, bottom). At week 4, vasoconstriction was significantly greater in the estrogen than in the placebo group (P<0.01 by multiple comparison). This difference, however, was no longer present at week 8. Acetylcholine-induced relaxation (stimulated NO release) was not significantly different at week 4 and 8, and not significantly different between the estrogen and placebo group, despite a trend to an estrogen-mediated improvement in dilatation response after 8 weeks (Figure 5).

NO Experiment II

Neither the mean concentration of plasma cholesterol during the experimental period nor the aortic accumulation of cholesterol was significantly different between the 2 groups after 4 weeks of individualized cholesterol feeding (data not shown).

Mononuclear Cell Adhesion

In rabbits not administered with L-NAME, aortic segments from estrogen-treated rabbits demonstrated a 50% decrease in cell binding compared with placebo-treated rabbits (P<0.05 by multiple comparison) (Figure 6). This difference, however, was significantly reduced by L-NAME administration.
(interaction, \(P=0.05\) by 2-way ANOVA). This resulted in no significant decrease in cell binding by treatment with estrogen in combination with L-NAME. L-NAME administration alone did not significantly change aortic cell binding.

Discussion

Atherosclerosis Experiment

Cholesterol feeding, in contrast to oil-enriched chow, significantly increased plasma and aortic cholesterol. The higher amount of dietary cholesterol given to the estrogen than to the placebo group suggests that estrogen would have significantly reduced plasma cholesterol levels had the rabbits not been individually fed cholesterol. The high levels of circulating cholesterol did not accumulate significantly in the undamaged aorta of the 2 groups within the first 4 weeks. Within the following 4 weeks, however, aortic cholesterol more than doubled in the placebo-treated rabbits. The inhibition of aortic cholesterol accumulation by estrogen, mediated independently of plasma cholesterol levels, suggests that estrogen in some way increases the resistance of the artery wall to atherosclerosis. Alternatively, estrogen may inhibit atherosclerosis by favorably affecting plasma lipoprotein patterns. Such changes are not equalized by the cholesterol clamping technique and were not measured in the current study. A number of studies, however, suggest that the beneficial effect of estrogen is either independent of or only partially explained by changes in plasma lipoproteins.

This present study confirms our previous finding that the direct antiatherogenic effect of estrogen is abolished by balloon catheter injury in cholesterol-fed rabbits. In previous experiments, however, the rabbits were euthanized 12 weeks after surgery. At that time, the balloon-injured area consists of areas denuded of endothelium as well as areas covered with regenerated endothelial cells that are irregularly shaped, lack alignment in the direction of the blood flow, and exhibit endothelial dysfunction. In this study, the effects of estrogen on the balloon-injured aorta were therefore separated into effects on reendothelialized and effects on deendothelialized tissue. The results suggest that the overall lack of effect of estrogen on balloon-injured tissue is the aggregate of a neutral effect of estrogen on reendothelialized tissue and a paradoxical atherogenic effect of estrogen on deendothelialized tissue. Furthermore, it demonstrates that these effects are also present at low levels of aortic cholesterol accumulation, adding further support to the idea that the different effects of estrogen on undamaged and balloon-injured aorta are explained by changes in the endothelial state rather than by a general attenuation of the antiatherogenic effect of estrogen at high levels of atherosclerosis.

NO Data

A paradoxical atherogenic effect as demonstrated in the atherosclerosis experiment has previously been observed in endothelium-denuded aorta of female compared with male rabbits and in balloon-injured (nonseparated) aorta of rabbits treated with estrogen+L-NAME. Thus, absence of endothelial NO, either by endothelial denudation or enzyme inhibition, may play a role for this previously unnoticed effect of estrogen/female sex on vascular tissue. NO is released from the endothelium both continuously (basal release), and in response to different agents (stimulated release) and acts as an endogenous vasodilator as well as an antiatherogenic molecule. In the present study, estrogen selectively increased basal release of NO. This is in accordance with previous reports in animals and humans, although the cause of this phenomenon is not known. It may not be explained by changes in eNOS protein level, as this would affect stimulated release of NO as well. Changes in eNOS protein level would also result in altered aortic eNOS activity which could not be demonstrated in the current experiment after 4 or 8 weeks. Thus, estrogen may affect basal NO release via alternative mechanisms. It has been suggested that estrogen via an interaction with its receptor induces a moderate elevation of free cytosolic calcium in endothelial cells, resulting in increased activity of the calcium-dependent eNOS. Such a moderate increase in calcium level would be masked by the more significant increase following stimulation with acetylcholine. Similarly, the increased eNOS activity in vivo would be masked in the current NOS assay, where calcium and all other known cofactors are added in excess. Alternatively, it has been suggested that estrogen inhibits superoxide anion production, a free radical able to react rapidly with NO leading to its inactivation.

Estrogen increased basal NO release from aortic rings only before differences in aortic cholesterol became apparent. This is in accordance with a previous study, where an increase in basal NO release by female sex was present before cholesterol feeding was initiated but not after 10 to 15 weeks of cholesterol feeding. A transiency in the effect of estrogen on basal NO release is further supported by the data for circulating nitrite/nitrate, showing an increase in the estrogen-treated groups after 1,2, and 4 but not after 8 weeks of cholesterol feeding. Thus, a possible involvement of NO in the direct antiatherogenic effect of estrogen may take place at an early stage of atherogenesis.

We have recently shown that long-term inhibition of NO synthesis significantly attenuates the direct antiatherogenic effect of estrogen in cholesterol-fed rabbits. NO has been demonstrated to decrease vascular cell adhesion molecule-1 (VCAM-1) expression in cultured endothelial cells, and to inhibit monocyte-endothelial cell interaction. A similar regulatory effect on VCAM-1 expression has been demonstrated for estrogen. Thus, a likely mechanism by which NO could mediate estrogen’s antiatherogenic effect is through a reduction in mononuclear-endothelial cell binding. Our data, showing a 50% decrease in cell binding of aortic segments from estrogen-treated rabbits compared with placebo-treated rabbits and an abolishment by this effect by simultaneous treatment with L-NAME, suggest (1) that the effect of estrogen on VCAM-1 expression is translated into a reduced mononuclear-endothelial cell binding in vivo and (2) that this effect of estrogen is mediated by its ability to increase NO production.

Conclusion

This study shows that the direct antiatherogenic effect of estrogen is already present after 8 weeks in the cholesterol-clamped rabbit model. The effect varied with the state of the
aortic endothelium, was paradoxically reversed in denuded areas, and was preceded by a transient increase in basal NO release and circulating nitrite/nitrate concentrations. This transient increase in NO production may be involved in the direct antiatherogenic effect of estrogen by in turn mediating a 50% reduction in mononuclear-endothelial cell binding, one of the earliest step in atherogenesis. Thus, these data add support to the notion that the endothelium and endothelial NO are involved in the mechanism by which estrogen inhibits atherosclerosis independently of plasma cholesterol levels.

Acknowledgments

These studies were supported by grants from the Danish Heart Foundation.

References

The Direct Antiatherogenic Effect of Estrogen Is Present, Absent, or Reversed, Depending on the State of the Arterial Endothelium: A Time Course Study in Cholesterol-Clamped Rabbits

Pernille Holm, Heidi L. Andersen, Malene R. Andersen, Elisabeth Erhardtsen and Steen Stender