Conjugated Equine Estrogens Inhibit Progression of Atherosclerosis but Have No Effect on Intimal Hyperplasia or Arterial Remodeling Induced by Balloon Catheter Injury in Monkeys

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Objectives. This study sought to determine the effects of estrogen treatment on atherosclerosis progression and the proliferative and structural responses of the atherosclerotic arteries to injury.

Background. Estrogen treatment suppresses the intimal response to arterial injury in nonatherosclerotic rodents and rabbits and inhibits the in vitro proliferation of smooth muscle cells. However, the effect of estrogen on the response of atherosclerotic arteries to transmural injury, as occurs in balloon catheter angioplasty in humans, is unknown.

Methods. Forty-six ovariectomized cynomolgus monkeys were fed an atherogenic diet for 30 months; 25 received 175 μg/day of conjugated equine estrogens, and 21 served as untreated control animals. All animals underwent balloon catheter injury of the left iliac artery. Subsets of animals underwent a necropsy study at 4, 7, 14 and 28 days after injury; injured and contralateral (uninjured) arteries were pressure-fixed and evaluated morphometrically.

Results. Estrogen treatment resulted in a 37% decrease (p < 0.05) in atherosclerosis (plaque area) in the uninjured artery. In response to injury, arterial cell proliferation increased at days 4 and 7, and intimal area was increased two- to threefold at day 28 (p < 0.05). Although estrogen treatment resulted in a trend toward decreased arterial cell proliferation at day 4, there was evidence of increased cell proliferation in both media and intima at day 7 (p < 0.05). However, there was no effect of estrogen treatment on intimal area or indexes of arterial remodeling in the injured artery at day 28 (p > 0.4).

Conclusions. In contrast to previous studies of nonatherosclerotic animals, the results indicate that in the circumstance of transmural injury to arteries of primates with preexisting atherosclerosis, estrogen does not suppress arterial neointimal or structural responses to injury.

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There is substantial clinical and experimental evidence that estrogen has a potent inhibitory effect on the initiation and progression of coronary artery atherosclerosis (1–9). Although the mechanism or mechanisms involved in the atheroprotective effects of estrogen are not completely elucidated, there is evidence that estrogen inhibits the intimal hyperplasia resulting from mechanical arterial injury in experimental animals (10–14) and inhibits the proliferation of cultured vascular smooth muscle cells (15,16). These findings have suggested that antiproliferative effects of estrogen may be involved in its atheroinhibitory effects and that estrogen may be useful in the prevention of angioplasty-associated restenosis in patients with coronary heart disease. However, previous studies of the effects of estrogen on the intimal response to injury have been conducted in nonatherosclerotic, nonprimate species. Although these models have contributed substantially to the understanding of the injury response, several interventions that are effective in preventing intimal hyperplasia in nonatherosclerotic animals (17,18) have been ineffective in humans in restenosis trials (19–21). This incongruence suggests the need for a model system that mimics the clinical situation in which true restenosis occurs: as a response to the injury of arteries with preexisting advanced atherosclerosis.

We have extensively used a monkey model to study the effects of sex hormones on the initiation and progression of atherosclerosis. We have found that surgically induced menopause (ovariectomy) results in accelerated progression of atherosclerosis (22) and that estrogen replacement therapy markedly inhibits this process (5,23). More recently, we characterized the structural and proliferative responses to mechanical arterial injury in atherosclerotic monkeys (24,25) and found that the processes closely resemble those of angioplasty-associated restenosis in humans. We present evidence that treatment with conjugated equine estrogens (CEE) has varying effects on the arterial cell proliferation response but no effect...
on neointimal and structural responses of the artery wall to balloon catheter injury in ovariectomized, atherosclerotic monkeys.

**Methods**

**Animals.** For the study, we used 46 female cynomolgus monkeys imported directly from Indonesia (Institut Pertanian Bogor, Bogor, Indonesia). For a total of 34 months, all animals were fed a moderately atherogenic diet (40% of calories as fat and 0.28 mg of cholesterol/kcal) (5). Monkeys lived in social groups consisting of 4 to 6 animals. All procedures involving animals were conducted in compliance with institutional animal care and use committee policies.

Monkeys were ovariectomized and consumed the atherogenic diet for a 4-month preexperimental period. They were assigned on the basis of a stratified randomization scheme with total plasma cholesterol and high density lipoprotein (HDL) cholesterol as stratification variables to one of two experimental groups: no treatment (estrogen-deficient control animals) (n = 21) or treatment with CEE (Premarin; Wyeth-Ayerst) (n = 25). These animals represent two of four experimental groups established for the purposes of a larger study of the effects of estrogens and progestins on primary atherosclerosis (23). CEE was administered in the diet continuously for 30 months. As in previous studies (26,27), the difference in caloric intake between monkeys and humans was used to calculate the appropriate dosage for the monkeys. This method adjusts for differences in both body size and metabolic rate; on this basis, a 4-kg monkey received 170 mg of CEE daily. Plasma concentrations of estradiol and estrone were measured with a radioimmunoassay to verify dosing resulted in plasma concentrations similar to those of women using CEE. The detection limit for these assays was 40 pmol/liter.

**Plasma lipids, lipoproteins.** Total plasma cholesterol (TPC) (28), HDL cholesterol (29) and triglyceride (30) levels were determined at 3-month intervals. Lipoprotein fractions were separated by ultracentrifugation and high performance liquid chromatography (31), and the cholesterol content of each fraction was quantified (32). Average low density lipoprotein (LDL) molecular weight was determined for each sample by including a trace amount of iodinated LDL of known molecular weight (33). At these same time points, plasma concentrations of apolipoprotein (apo)B (34) and apo A-I (35) were determined by enzyme-linked immunosorbent assay, and HDL subfractional size heterogeneity was assessed using polyacrylamide gel electrophoresis (4% to 30%, Pharmacia).

**Arterial injury protocol.** Near the end of the treatment period, all animals underwent balloon injury of the left common iliac artery. The right common iliac served as an uninjured control vessel. Animals were anesthetized and administered 100 units/kg heparin intravenously. A 3F balloon catheter was introduced into the left femoral artery and advanced to the distal aorta, where it was inflated and retrieved. This was done three times, followed by removal of the catheter and closure of the incision.

Arterial response to angioplasty was studied in subsets of five to seven animals from each treatment group that were killed at days 4, 7, 14 and 28 after angioplasty. To label proliferating cells (those entering S phase), bromodeoxuryidine (BrdU) (45 mg/kg intramuscularly) was administered at 18 h and again at 6 h before the necropsy.

**Necropsy, histopathology, histomorphometry.** At necropsy, animals were anesthetized deeply with pentobarbital (30 mg/kg intravenously), and the cardiovascular system was flushed with lactated Ringer’s solution. The distal abdominal aorta was cannulated, and the iliac arteries were perfused at 100 mm Hg in situ with lactated Ringer’s solution until the perfusate was clear, followed by perfusion with 10% neutral buffered formalin for 1 h. The iliac arteries were excised and immersion-fixed in formalin for an additional 24 h. Each common iliac artery was cut into five sequential rings and embedded in paraffin. Cross sections of each ring were stained with Verhoeff–van Gieson’s stain and projected onto a digitizing pad for measurement of lumen, medial and intimal areas and the areas encompassed by internal and external elastic laminae. Values for each iliac artery were determined by averaging measurement values for the five adjacent rings.

**Cell proliferation.** Deparaffinized sections were stained with a monoclonal antibody against BrdU (1:20 dilution; Boehringer-Mannheim) and incubated overnight at 4°C. A biotinylated secondary antibody was applied and localized using the ABC reaction. Sections were counterstained and examined at 600×. Proliferating cells, identified by dark brown nuclear staining, were counted and localized to the intima, media or adventitia of each section. A cell proliferation index (%) was calculated by dividing the number of labeled nuclei by the number of total nuclei and multiplying the result by 100. Mean proliferation indices were calculated as the average of the values from the five sections from each iliac artery.

**Cell types.** For identification of cell types, we used the antibodies (24) smooth muscle cell alpha-actin (Boehringer-Mannheim), endothelial cell von Willebrand factor (Dako) and macrophage CD68 (Dako).

**Statistical analyses.** Two-way analysis of variance (treatment [nonrepeated measures] × injury [repeated measures]) was used to assess cell proliferation and response to injury at each time point (4, 7, 14 and 28 days). Data underwent square-root transformation when necessary to reduce skewness and equalize between-group variances.

### Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>apo</td>
<td>apolipoprotein</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxuryidine</td>
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<tr>
<td>CEE</td>
<td>conjugated equine estrogens</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<td>LDL</td>
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<td>TPC</td>
<td>total plasma cholesterol</td>
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<td>VLDL</td>
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Results

Animals. Two animals died during the time between balloon injury and necropsy of causes unrelated to the study. The arteries of 2 animals developed occlusive thrombi after balloon injury. All data from these animals were excluded from analyses. Data provided here represent the 19 untreated control animals and 23 animals treated with CEE. Plasma concentrations of estrone and estradiol were 256 ± 56 and 410 ± 65 pmol/liter, respectively, for CEE-treated animals and were undetectable in control animals.

Plasma lipoproteins and atherosclerosis. TPC levels were decreased 14% (p < 0.05) and triglyceride levels were increased 135% (p < 0.05) in estrogen-treated animals relative to control animals. Plasma HDL cholesterol concentrations did not differ between groups. Lipoprotein data have been given previously (23). In brief, estrogen treatment resulted in a 53% decrease in VLDL cholesterol, a 16% decrease in average LDL molecular weight, a 30% decrease in the HDL 2b subfraction and a 63% increase in the HDL 3b subfraction (for all, p < 0.05).

Arterial morphology. Plaques in uninjured iliac arteries were composed primarily of smooth muscle cells, macrophages and extracellular matrix underlying an endothelial cell monolayer (24). There were frequent areas of calcification and necrosis as well as areas of microvascular ingrowth.

Morphologic descriptors of the structural sequelae of arterial balloon injury in monkeys have been described previously (24,25). In brief, acute plaque fracture, dissection and thin nonocclusive thrombi were common at days 4 and 7. By 14 days, fractures and dissections had begun to fill in with neointima, which was typically composed primarily of smooth muscle cells and extracellular matrix. The neointima thickened markedly between days 14 and 28 and was histologically distinct from the underlying preexisting atherosclerosis, with few macrophages and vasa, no calcification and a more homogeneous and pale extracellular matrix than the atherosclerotic plaque. There were no differences between treated and untreated animals in the cellular composition or morphology of the neointima.

Morphometric descriptors. Atherosclerotic plaque area was reduced 37% (p < 0.05) in the right (uninjured) iliac artery of estrogen-treated animals relative to untreated control animals.

Balloon injury resulted in increased intimal area at days 14 (p < 0.05) and 28 (p < 0.003). However, the magnitude of this increase did not differ between treatment groups at any time point (for all, p > 0.4 for group × injury interaction and p > 0.2 for main effect) (Fig. 1). Mean artery size (external elastic lamina area) was transiently increased at days 4 (p < 0.004) and 7 (p < 0.03) (Fig. 2). Artery size was increased in estrogen-treated animals relative to control animals at day 7 (p < 0.05, group × injury interaction) (Fig. 2). Increases in artery size at days 4 and 7 were paralleled closely by increases in lumen size (Fig. 3).

Cell proliferation. Intimal thickening was preceded by cellular proliferation (increased labeling by BrdU) throughout the injured artery wall (intima, media and adventitia) at days 4 and 7. This wave of proliferation had completely subsided by day 14.

Estrogen treatment resulted in a tendency toward a decrease in cell proliferation at day 4 (Fig. 4), although in no case did these differences reach statistical significance (for main effect, p = 0.07, 0.10 and 0.34; for group × injury interaction,
p = 0.17, 0.56 and 0.74). At day 7 (Fig. 4), however, although cell proliferation was decreased in the adventitia (p < 0.004 for main effect and p < 0.09 for group × injury interaction), it was increased in media (p < 0.004 for group × injury interaction) and intima (p < 0.04 for group × injury interaction). At days 14 and 28, cell proliferation in response to injury had subsided, and labeling rates had returned to very low levels. There were no effects of estrogen treatment at days 14 and 28 (all p > 0.2) (data not shown).

**Discussion**

The two major findings of this study were that treatment of ovariectomized monkeys with CEE (equivalent to a woman’s dosage of 0.625 mg/day) for 30 months resulted in a reduced extent of diet-induced iliac artery atherosclerosis yet had no
persistent effects on the structural or hyperplastic responses of atherosclerotic iliac arteries to transmural balloon catheter–induced injury.

Pathogenetic considerations. Although the responsible stimuli are uncertain, the migration and proliferation of smooth muscle cells appear to be prominent features of the pathogenesis of both atherosclerosis and injury-induced intimal hyperplasia. Whether atherosclerosis- and injury-associated cell proliferation are pathogenetically similar, however, is unclear. There is evidence that estrogen treatment has similar inhibitory effects on the progression of atherosclerosis (1–9), the myointimal proliferation associated with mechanical arterial injury in nonatherosclerotic animals (10–14) and the in vitro proliferation of smooth muscle cells (15,16).

Our data are consistent with these earlier findings in that we also observed a tendency toward a decrease in arterial cell proliferation rate in estrogen-treated animals immediately after injury (day 4). However, at day 7, we found evidence for a reversal of this effect, with cell proliferation actually increased in the media and intima of estrogen-treated animals. These findings indicate that in primates with preexisting atherosclerosis, although estrogen treatment may inhibit cell proliferation very early after angioplasty, estrogen may simply delay the proliferative response while having no lasting effect on clinically relevant end points such as accumulation of neointimal mass, arterial remodeling and lumen stenosis. In addition, these data suggest that the use of cell proliferation as an index of neointimal accumulation may be misleading, particularly if observations are made at a single time point.

Methodologic considerations. Although the results of the present study agree with those addressing effects of estrogen on atherosclerosis, they seem to conflict with those of the effects of estrogen on the intimal response to arterial injury. It seems likely that this apparent contradiction is accounted for by substantial differences in experimental design.

One difference in our study design is in the type of estrogen that we used. In previous studies of nonatherosclerotic rodents and rabbits, the effects of parenterally administered estradiol were studied. In our study, we addressed the effects of oral CEE, the form of estrogen replacement most commonly prescribed to postmenopausal women in North America. CEE is a mixture of at least nine sulfated estrogen esters extracted from the urine of pregnant mares. Although its metabolism and the determinants of its target tissue activity are very complex and still incompletely understood, CEE has been prescribed for symptoms of estrogen deficiency for >50 years. The daily dose equivalent of CEE used in the present study, 0.625 mg, is known to have potent estrogenic activity in the liver, bone, uterus, mammary gland and cardiovascular system of both women and female monkeys and to markedly inhibit atherosclerosis (23) and promote endothelium-dependent vasodilation in monkeys (36). Thus, it seems unlikely that the lack of effect on arterial response to injury can be explained by

Figure 4. BrdU-labeled cells (expressed as percent of total cells) (mean ± SEM) at days 4 and 7 after injury in adventitia, media and intima of injured and uninjured iliac arteries of ovariectomized monkeys treated with CEE (estrogen) and untreated control monkeys (control). Injury resulted in increased cell division in all layers at days 4 and 7 (p < 0.001). At day 4, estrogen treatment resulted in a tendency toward decreased cell proliferation in intima (p < 0.07) and media (p < 0.10). At day 7, estrogen treatment resulted in decreased cell proliferation in adventitia (p < 0.004) but increased cell proliferation in intima (p < 0.04) and media (p < 0.004).
a lack of activity of CEE at the level of the artery. However, we cannot rule out the possibility that the level of activity was insufficient to influence the response to injury and that a higher dose may have been effective.

Another important difference in our study design is that in previous studies, nonatherosclerotic rodents or rabbits were used, which are models that may have limited relevance to the clinical situation in which the response of vessels with advanced atherosclerosis is involved. It is now clear that compounds that are effective in inhibiting the myointimal response to arterial injury in nonatherosclerotic animals typically have been ineffective in preventing arterial restenosis in clinical trials (17–21). The reason or reasons for this incongruence are unclear. Among the possible explanations are species differences in the regulation of smooth muscle migration and proliferation (37) or differences in the responses of atherosclerotic and nonatherosclerotic arteries (38–40). Furthermore, the end points typically addressed in studies with animals (i.e., neointimal proliferation and mass) may be minor determinants of angioplasty-associated restenosis in humans (41–43).

For these reasons, we studied the arterial response to injury in monkeys with preexisting diet-induced atherosclerosis. In this model, the injury response shares many morphologic characteristics with the response observed in humans (24,25), including an acute increase in artery and lumen diameter, plaque fracture or dissection, mural thrombus deposition and medial fracture or delamination. Subsequently, in some, although not all, subjects, the acute increases in artery and lumen diameter are lost within days of injury, probably due to delayed spasm of the artery wall (24,41). Interestingly, this acute injury-related increase in artery and lumen size persisted in estrogen-treated, but not control, animals at day 7, although it was lost thereafter and thus had no effect on long-term indices of artery structure. We can only speculate whether this short-lived phenomenon was related to the well known vasorelaxant properties of estrogens.

After these acute changes, neointima begins to appear. It is interesting, and perhaps relevant to the findings reported here, that neointimal accumulation occurs between 14 and 28 days after injury, which is after the proliferative response has largely subsided. Whether neointimal accumulation results in lumen encroachment, however, depends primarily on the degree of arterial remodeling that occurs simultaneously in response to injury (25). In the present study, estrogen treatment had no apparent influence on remodeling and, consequently, no effect on lumen caliber at 28 days after injury. Therapeutic implications. Efforts to prevent angioplasty-induced restenosis have targeted, almost exclusively, the intimal hyperplastic response. However, this may be only one of many determinants of restenosis. In restenosis, as in atherosclerosis, there may be a compensatory enlargement (remodeling) of the artery that accompanies the growth of the neointima and results in an increase in lumen diameter (25,43). It seems probable that the presence or absence of restenosis depends on interrelationships between lesion growth and arterial remodeling. Furthermore, these interrelationships may be influenced by genetic factors (37); the presence, absence or characteristics of the underlying atherosclerotic process (38–40); local hemodynamic forces (44); and other, undiscovered factors to determine whether restenosis occurs.

Conclusions. Although the effects of estrogen on atherosclerosis-related remodeling require further investigation, the data presented here indicate that although CEE treatment inhibits the progression of diet-induced atherosclerosis, it has no effect on the short-term (28-day) neointimal or remodeling responses of the artery to balloon catheter injury in a nonhuman primate model.

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References


