Conjugated Equine Estrogens Alone, but Not in Combination With Medroxyprogesterone Acetate, Inhibit Aortic Connective Tissue Remodeling After Plasma Lipid Lowering in Female Monkeys

Thomas C. Register, Michael R. Adams, Deborah L. Golden, Thomas B. Clarkson

Abstract—The objective of this study was to determine the arterial responses to plasma lipid lowering alone or in combination with (1) estrogen replacement therapy or (2) hormone replacement therapy in surgically postmenopausal female monkeys with preexisting atherosclerosis. Eighty-eight female cynomolgus macaques were ovariectomized, fed an atherogenic diet for 24 months, and then assigned by randomized stratification into 4 groups. One group (baseline, n=20) was necropsied at the end of the atherogenic diet period; the remaining 3 groups were fed a plasma lipid–lowering diet (regression) for 30 months. These regression groups were control (diet only), CEE (receiving conjugated equine estrogens alone), and CEE+MPA (receiving CEE and continuous medroxyprogesterone acetate). A previous report described coronary artery functional and histological results; the present report describes biochemical and histological results from the abdominal aorta. Aortic plaque size was not different between groups, similar to previous findings in the coronary arteries. Aortic cholesterol content (milligrams per gram lipid-free dry weight) was lower in the regression groups compared with baseline, both for free cholesterol (mean, control=19.1, CEE=15.7, CEE+MPA=14.4, and baseline=32.7; P<0.001) and for esterified cholesterol (mean, control=18.9, CEE=15.4, CEE+MPA=14.2, and baseline=58.7; P<0.001). This cholesterol efflux could lead to increased plaque stability without changing the physical size of the lesion. Alterations in aortic connective tissue composition were observed in the regression groups. When expressed as a percentage of the lipid-free tissue weight, the aortic elastin content of the control (mean=14.9) and the CEE+MPA (mean=14.0) groups was lower than that of the baseline group (mean=19.0), which was not different from that of the CEE group (mean=15.8). Aortic collagen content, as estimated by hydroxyproline content per milligram of lipid-free tissue, was higher in the control group (mean=67.4) and the CEE+MPA group (mean=66.1) than in the baseline group (mean=56.2; P<0.05). Collagen content of the CEE group (mean=58.9) was not different from that of the baseline group. When the regression groups were considered separately, the aortic collagen content of the CEE group was lower than that of the control group (P<0.05) and tended to be lower than that of the CEE+MPA group (P=0.10), suggesting that CEE therapy (but not CEE+MPA) inhibits potentially detrimental connective tissue alterations that accompany lesion regression. These results have implications for combinations of lipid-lowering and hormone replacement therapies in relation to vascular remodeling and abdominal aortic aneurysm development. (Arterioscler Thromb Vasc Biol. 1998;18:1164-1171.)

Key Words: collagen ■ elastin ■ plaque stability ■ aortic aneurysm

Numerous studies have shown that atherosclerosis progression can be retarded, and in some cases at least partially reversed, when the atherogenic stimulus is reduced or removed. Regression initiated by lowering of plasma lipid concentrations is generally characterized by efflux of lipid from the lesion and repair of the injured areas, with the end result depending on the degree of injury initially present. Minimally affected atherosclerotic lesions may be fully regresisible, whereas more advanced lesions may undergo a process in which loss of lipid from the lesion is accompanied by necrosis or fibrosis. Most of the studies examining atherosclerosis regression to date have been performed in males, and a number of these investigations have utilized nonhuman primates owing to the pathological and pathophysiological similarity of their atherosclerotic lesions to those found in human beings.7-9

Generally, women do not develop clinically significant coronary heart disease until some time after menopause, perhaps owing to the antiatherogenic effects associated with normal ovarian function.68 Despite this protection during the premenopausal years, coronary heart disease is still the leading cause of death in women >60 years of age. Estrogen

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replacement therapy with and without opposing progestins has been shown to extend this "female protection" after the cessation of ovarian function due to surgical or natural causes in both a nonhuman primate model and in women. The beneficial effects of estrogen were only partially accounted for by its effects on circulating lipids in these studies, suggesting that direct effects on the artery wall also were important. One potential site of action for estrogen is arterial matrix production, and several investigators have suggested that estrogen may influence arterial collagen synthesis in vivo and in vitro.

The present study was designed to determine whether estrogen replacement therapy alone or with an added progestin (hormone replacement therapy) had a beneficial effect on atherosclerosis when combined with plasma lipid lowering. A previous report described the results of this study with respect to plasma lipid and lipoprotein concentrations, morphometrically determined coronary artery atherosclerosis, and coronary artery vasomotion. The current report describes the effects of these hormone therapies on the arterial biochemical changes associated with regression, especially with respect to connective tissue composition.

Methods

Animals

The subjects of this study were 120 feral adult female cynomolgus monkeys (Macaca fascicularis) imported from Indonesia (Charles River Primates, Port Washington, NY). Of these, 88 animals were available for analysis at the end of the study owing to losses from causes unrelated to the experimental manipulations (primarily trauma or gastrointestinal disorders) as previously described. At the initiation of the study, animals ranged in age from 5 to 13 years as estimated from dentition and were not pregnant. Animals were housed in social groups of 4 to 8 monkeys each. Bilateral ovariectomy was performed on all animals at the start of the atherosclerosis induction period (see Figure 1 for trial design). All animals were then fed a moderately atherogenic diet containing 43% of calories from fat and 0.44 mg cholesterol per kcal for 24 months to induce atherosclerosis (Figure 1). At the end of the induction phase, the animals were assigned to 1 of 4 groups by using a method of stratified randomization. The groups were stratified by the (1) TPC area encompassed by the IEL, the IEL area: IELA (IEL/2), and the (2) bone density measured 8 months before treatment onset (because a secondary end point of the study was to determine the effects of estrogen replacement therapy and hormone replacement therapy on bone density), and (3) time since ovariectomy/initiation was used to normalize the length of time on the diet. The mean age of the animals at the initiation of the study was 8.75 years, with no difference in age between the 4 groups. Group 1 animals were necropsied at the end of the induction period (baseline group, n = 20). The remaining 3 groups of animals were fed a plasma lipid–lowering diet consisting of 0.05 mg cholesterol per kcal (equivalent to human consumption of 100 mg/d) and containing 30% of calories as fat for 30 months (treatment phase). These regression groups consisted of (1) an untreated control group (control), (2) a group receiving CEE (Premarin, Wyeth-Ayerst Laboratories), and (3) a group receiving CEE plus continuous MPA (the MPA was Cycrin, Wyeth-Ayerst Laboratories). For 8 months of the 30-month treatment period, the CEE and CEE+MPA groups received 7.2 μg of CEE per monkey per day. For 22 of the 30 months, the dose of CEE was increased to 166 μg per monkey per day to be equivalent to a human dose of 0.625 mg/d. Throughout the 30-month treatment phase, the CEE+MPA group received 650 μg per monkey per day of MPA, equivalent to a woman’s dose of 2.5 mg/d. Hormones were administered twice daily in the diet. Regression animals were necropsied after 30 months of treatment (54 months total). All procedures involving animals were conducted in compliance with state and federal laws, the standards of the Department of Health and Human Services, and guidelines established by the Institutional Animal Care and Use Committee of the Wake Forest University Medical School.

Aortic Measurements

At necropsy, the abdominal aorta was carefully cleaned of adventitial tissue, opened longitudinally along the posterior surface, and sectioned into segments for histological evaluation and determination of chemical composition. Five separate segments of the abdominal aorta from proximal (No. 1, below the renal arteries) to distal (No. 5, above the iliac arteries) areas were obtained. Sections 1, 3, and 5 were used for histology and sections 2 and 4 were frozen for biochemical analyses. Results obtained from sections 3, 4, and 5 are presented here.

Histological Analysis

Sections 3 and 5 were cut perpendicular to the long axis of the longitudinally opened abdominal aorta, pinned out on cardboard, and then fixed with 10% neutral buffered formalin. One histological section was made from each block and stained with Verhoeff–van Gieson’s stain. These sections were projected, and the cross-sectional area of intimal lesions (which were composed of fatty streaks, plaque, or both) were measured by using a digitizer. Atherosclerosis extent was expressed as the cross-sectional area of intimal lesions in millimeters squared. The IELA lengths of the opened sections were also measured. Artery size was estimated by using the IELA length as a circumference for mathematical derivation of the area encompassed by the IELA, the IELA area: IELA = π(IELA/2)². Lumen area was then estimated by subtracting the intimal area from the IELA.

Aortic Lipid Composition

The segments used for chemical composition studies (section 4 as described above) were ∼1 cm² and weighed ∼60 mg. These sections were pinned flat on a dissection board and photographed for subsequent determination of surface areas by using a Summagraphs morphometer and software (Woods Hole Educational Associates). Wet weights were obtained from tissue that had been blotted to

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**Selected Abbreviations and Acronyms**

- AAA = abdominal aortic aneurysm
- CEE = conjugated equine estrogen
- IELA = internal elastic lamina area
- LFDW = lipid-free dry weight
- MPA = medroxyprogesterone acetate
- TPC = total plasma cholesterol

**Table 1. Experimental design.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Randomization</th>
<th>Necropsy</th>
<th>Baseline Group (n = 20)</th>
<th>Control Group (n = 25)</th>
<th>CEE Treatment Group (n = 22)</th>
<th>CEE + MPA Treatment Group (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 Months PROGRESSION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 Months REGRESSION</td>
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</tbody>
</table>
remove surface liquid. Lipids were extracted from these tissues with 20 volumes (vol/vol) of chloroform-methanol (3:1, vol/vol). The LFDWs were determined by drying in vacuo to a constant weight. Tissue cholesterol content (free and esterified) was determined by the method of Rudel and Morris.18

Aortic Calcium Content
The lipid-free dry artery was rehydrated and decalcified with 0.1 mol/L HCl at 4°C for 7 days. Calcium content of the acid extract was determined using Arsenazo III reagent and protocols supplied with Roche Reagents’ “Reagent for calcium” (Roche Diagnostic Systems), except that all measurements were carried out using a microtiter plate reader (Biotek EL-340) at 630 nm with background correction at 450 nm. Calcium standards were prepared and assayed under the same conditions as were samples (in 0.1 mol/L HCl). The decalcified, delipidated tissue was again dried to a constant weight (decalcified LFDWs).

Aortic Collagen and Elastin Contents
Tissues were rehydrated in deionized water at 4°C for several days, and collagen was solubilized from the tissue by hot alkali extraction at 98°C in 0.1N NaOH for 50 minutes in a shaking water bath.19 The insoluble material (elastin) was separated from the soluble collagen by centrifugation, washed with 0.1 mol/L NaOH and then with deionized water, and dried under vacuum to a constant weight. The extracted collagen fraction was acid hydrolyzed in 6 mol/L HCl at 4°C for 7 days. Calcium content of the acid extract was determined using Arsenazo III reagent and protocols supplied with the method of Bergman and Loxley20 to estimate collagen content.

Expression of Data
Angiochemical measurements were expressed on a concentration basis (milligrams per gram of wet or lipid-free dry aorta) and on an area basis (milligrams per centimeter squared of flat aorta). In general, the use of wet weight of the tissue tends to underestimate the amount of a component per unit tissue, as increases in lipid and cell contents of the atherosclerotic aorta increase the wet weight of the tissue. The weight of the tissue after lipid extraction (LFDW, in grams) gives a more accurate estimate of the unit of tissue but is subject to change as a result of connective tissue changes in the artery. The surface area of a tissue section (in millimeters squared) is less likely to be altered by chemical changes but can be affected by vascular remodeling (shrinkage or enlargement) and is less accurately measured, especially in tissue that has been previously frozen.

Statistical Analysis
Data were analyzed by ANOVA. Owing to the complicated nature of the studies investigating the biochemistry of regression of atherosclerosis, ANOVA was carried out using 2 separate approaches. Initially, all data were analyzed by a 1×4 ANOVA that included the baseline group as well as the 3 regression groups, a design that allowed for a comparison of the chemical composition of the 3 regression groups with that of the baseline animals. The purpose of the baseline group, a subgroup of animals randomly sampled from the entire study population before initiation of the lipid-lowering diet, was to provide a means of assessment of the extent of atherosclerosis before regression commenced. In effect, this provides a means to estimate alterations occurring in the artery during the period of lipid lowering. In addition, data from only the regression groups, which were subjected to dietary manipulation for 30 more months, were analyzed by a 1×3 ANOVA excluding the baseline animals from the analysis. This allowed for a better assessment of differences between the individual regression groups. Variables not meeting homogeneity of variance assumptions were subjected to logarithmic transformation. Means and SDs of the data are presented. Post hoc analysis was performed by multiple-comparison tests with Bonferroni-adjusted significance levels for the number of tests in each analysis. There were 6 post hoc comparisons in the 1×4 ANOVA as follows: baseline versus (1) control, (2) CEE, and (3) CEE+MPA; control versus (4) CEE and (5) CEE+MPA; and (6) CEE versus CEE+MPA. There were 3 post hoc comparisons in the 1×3 ANOVA as follows: (1) control versus CEE, (2) control versus CEE+MPA, and (3) CEE versus CEE+MPA. Significance levels given in the text are the result of post hoc tests. Significance levels of the ANOVA are presented in the tables.

Results
Plasma Lipid Concentrations
Details of the plasma lipid and lipoprotein compositions of the different groups during the progression and regression phases of the project have been described.17 In brief, the atherogenic diet induced marked hypercholesterolemia, resulting in mean TPC concentrations of ~16.8 mmol/L. There were no differences between experimental groups in plasma lipid or lipoprotein concentrations before the onset of the regression diet and hormone treatment. The regression diet resulted in a lowering of TPC levels of 3.90 to 4.15 mmol/L.

Aortic Morphometric Analysis
Results of histomorphometric analysis of sections of the abdominal aorta immediately proximal (section 3) and distal (section 5) to the segment used for biochemical analysis (section 4) are shown in Table 1. Plaque size and lumen area were not different between groups for either section, although there was a trend toward increased plaque size and lumen area in the proximal section of the control group. Artery size (as indicated by intimal area; LA, lumen area. Results are mean±SD. *Significantly different from baseline in P<0.05.

Table 1. Abdominal Aortic Histomorphometric Results

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Control</th>
<th>CEE</th>
<th>CEE+MPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal section</td>
<td>n=20</td>
<td>n=25</td>
<td>n=21</td>
<td>n=21</td>
</tr>
<tr>
<td>IA, mm²</td>
<td>1.23±0.70</td>
<td>1.97±1.39</td>
<td>1.40±1.01</td>
<td>1.33±0.87</td>
</tr>
<tr>
<td>IELA, mm²</td>
<td>4.40±1.11</td>
<td>6.22±2.51</td>
<td>5.09±1.42</td>
<td>4.92±1.61</td>
</tr>
<tr>
<td>LA, mm²</td>
<td>3.17±0.93</td>
<td>4.25±1.62</td>
<td>3.69±1.28</td>
<td>3.59±1.15</td>
</tr>
</tbody>
</table>

| Distal section   | n=20     | n=24    | n=20 | n=20   |
| IA, mm²          | 1.24±0.93| 1.84±1.19| 1.49±0.98| 1.38±0.74|
| IELA, mm²        | 4.49±1.32| 5.31±2.38| 5.17±1.82| 4.57±1.35|
| LA, mm²          | 3.25±0.80| 3.47±1.66| 3.68±1.56| 3.19±1.11|

IA indicates intimal area; LA, lumen area.

Table 2. Abdominal Aortic Histomorphometric Statistics

<table>
<thead>
<tr>
<th>End Point</th>
<th>ANOVA 1×4 (Baseline and Treatment Groups)</th>
<th>ANOVA 1×3 (Treatment Groups Only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal section</td>
<td>IA, mm²</td>
<td>F[3,63]=2.38, P=0.08</td>
</tr>
<tr>
<td></td>
<td>IELA, mm²</td>
<td>F[3,63]=4.24, P=0.01</td>
</tr>
<tr>
<td></td>
<td>LA, mm²</td>
<td>F[3,63]=2.68, P=0.052</td>
</tr>
<tr>
<td>Distal section</td>
<td>IA, mm²</td>
<td>F[3,80]=1.52, NS</td>
</tr>
<tr>
<td></td>
<td>IELA, mm²</td>
<td>F[3,80]=1.12, NS</td>
</tr>
<tr>
<td></td>
<td>LA, mm²</td>
<td>F[3,80]=0.56, NS</td>
</tr>
</tbody>
</table>

IA indicates intimal area; LA, lumen area.
were observed when the regression groups were considered separately (Table 2).

**Aortic Lipid Composition**
The biochemical composition of the abdominal aortas of each group are shown in Table 3, and statistical results obtained from these data are presented in Table 4. After 2 years of the atherogenic diet, the abdominal aortas of the baseline group had very high cholesterol contents (≈14 μg cholesterol per milligram wet weight) compared with normal arteries (≈1 to 2 μg cholesterol per milligram wet weight) as previously described.\(^{5,6,21}\) All 3 regression groups had reduced aortic cholesterol contents relative to the baseline group, as shown by 1×4 ANOVA (Table 4), whether expressed per unit of tissue wet weight, per unit of tissue LFDW (Figure 2), or on an area basis (all \(P<0.05\)). The abdominal aortic cholesterol levels for the regression groups were ≈40% of those in the baseline group. No differences between the individual regression groups were found, as shown by 1×3 ANOVA (Table 4). The differences in TPC content between the baseline and regression groups reflected lower levels of both free cholesterol and esterified cholesterol in the regression groups. No effects of estrogen replacement therapy (CEE) or hormone replacement therapy (CEE+MPA) were observed for total, free, or esterified cholesterol contents in the abdominal aorta. The lower TPC content in the regression groups resulted in large part from a reduction in esterified cholesterol levels.

**Aortic Connective Tissue and Mineral Contents**
Abdominal aortic connective tissue compositions were different between groups. Elastin content of the abdominal aorta was altered by the lipid-lowering regimen, as shown by 1×4 ANOVA (Table 4), because the control and CEE+MPA groups had a lower proportion of elastin per unit weight or area than did the baseline group (both \(P<0.05\), Figure 3). The elastin content of the aorta of the CEE group was not different from that of baseline (\(P>0.10\)). No statistical differences were observed when the 3 regression groups were considered separately (all \(P>0.10\)).

Aortic collagen content was different between groups, as shown by both 1×4 and 1×3 ANOVAs (Table 4 and Figure 4). When all 4 groups were analyzed together, aortic hydroxyproline content (as a measure of aortic collagen) expressed per unit LFDW was higher in the control and CEE+MPA groups than in the baseline group (both \(P<0.05\)). Hydroxyproline content of the CEE group was not different from the baseline group (\(P>0.10\)). When the 3 regression groups were analyzed by 1×3 ANOVA, aortic collagen content (milligrams per gram LFDW) of the CEE groups was lower than that of the control group (\(P<0.05\) and tended to be lower than that of the CEE+MPA group (\(P=0.10\)). No differences among groups were found when hydroxyproline was expressed on an area basis, although there was a trend toward an increase in the control group relative to baseline (\(P=0.10\)).

### TABLE 3. Experimental Data From Abdominal Aortas of Individual Groups

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Control</th>
<th>CEE</th>
<th>CEE+MPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC</td>
<td>n=20</td>
<td>n=25</td>
<td>n=22</td>
<td>n=21</td>
</tr>
<tr>
<td>mg/g wet weight</td>
<td>14.3±6.70</td>
<td>6.33±5.19</td>
<td>5.73±3.76</td>
<td>5.32±3.14</td>
</tr>
<tr>
<td>mg/g LFDW</td>
<td>91.5±49.8</td>
<td>36.8±31.5</td>
<td>31.1±19.7</td>
<td>28.6±17.0</td>
</tr>
<tr>
<td>mg/cm²</td>
<td>9.74±5.83</td>
<td>3.89±3.52</td>
<td>3.34±2.81</td>
<td>3.94±2.20</td>
</tr>
<tr>
<td>Percent esterified</td>
<td>n=20</td>
<td>n=24</td>
<td>n=22</td>
<td>n=21</td>
</tr>
<tr>
<td>Elastin</td>
<td>58.5±14.3</td>
<td>42.6±11.5</td>
<td>45.6±11.4</td>
<td>45.5±12.9</td>
</tr>
<tr>
<td>Cholesterolester</td>
<td>n=20</td>
<td>n=24</td>
<td>n=22</td>
<td>n=21</td>
</tr>
<tr>
<td>mg/g wet weight</td>
<td>9.08±5.26</td>
<td>3.23±3.09</td>
<td>2.83±2.30</td>
<td>2.63±2.05</td>
</tr>
<tr>
<td>mg/g LFDW</td>
<td>58.7±38.8</td>
<td>18.9±19.0</td>
<td>15.4±11.9</td>
<td>14.2±11.2</td>
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<tr>
<td>mg/cm²</td>
<td>6.26±4.26</td>
<td>2.02±2.10</td>
<td>1.68±1.70</td>
<td>1.49±1.40</td>
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<tr>
<td>Free cholesterol</td>
<td>n=20</td>
<td>n=24</td>
<td>n=22</td>
<td>n=21</td>
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<tr>
<td>mg/g wet weight</td>
<td>5.19±1.96</td>
<td>3.29±2.16</td>
<td>2.90±1.57</td>
<td>2.68±1.21</td>
</tr>
<tr>
<td>mg/g LFDW</td>
<td>32.7±14.1</td>
<td>19.1±12.9</td>
<td>15.7±8.4</td>
<td>14.4±6.5</td>
</tr>
<tr>
<td>mg/cm²</td>
<td>3.48±1.84</td>
<td>2.01±1.46</td>
<td>1.65±1.15</td>
<td>1.46±0.85</td>
</tr>
<tr>
<td>Calcium</td>
<td>n=20</td>
<td>n=25</td>
<td>n=22</td>
<td>n=21</td>
</tr>
<tr>
<td>mg/g wet weight</td>
<td>2.74±7.09</td>
<td>2.80±3.38</td>
<td>4.45±8.61</td>
<td>1.46±1.80</td>
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<tr>
<td>mg/g LFDW</td>
<td>14.9±38.7</td>
<td>14.8±16.9</td>
<td>20.9±36.1</td>
<td>7.84±9.87</td>
</tr>
<tr>
<td>mg/cm²</td>
<td>2.24±6.09</td>
<td>1.62±1.93</td>
<td>2.75±5.47</td>
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<td>n=25</td>
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<tr>
<td>mg/g LFDW</td>
<td>56.2±8.9</td>
<td>67.4±12.5</td>
<td>58.9±13.1</td>
<td>66.1±9.5</td>
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<td>μg/mm²</td>
<td>5.75±1.19</td>
<td>6.81±1.64</td>
<td>5.95±1.74</td>
<td>6.54±1.52</td>
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<td>Elastin</td>
<td>n=20</td>
<td>n=25</td>
<td>n=22</td>
<td>n=21</td>
</tr>
<tr>
<td>% LFDW</td>
<td>19.0±3.4</td>
<td>14.9±4.7</td>
<td>15.8±4.5</td>
<td>14.0±3.8</td>
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<tr>
<td>μg/mm²</td>
<td>19.9±7.2</td>
<td>14.8±4.5</td>
<td>15.8±4.3</td>
<td>13.7±3.9</td>
</tr>
</tbody>
</table>

Results are mean±SD.
The calcium content of the abdominal aortas within groups was extremely variable, because some artery sections contained complicated lesions with calcified areas. No differences were found between any groups with respect to aortic calcium content, whether expressed on a weight or area basis, or when analyzed in the regression groups only (all \( P > 0.10 \)).

### Discussion

Regression of atherosclerosis can be defined in several ways: (1) a reduction in the size of intimal lesions in an atherosclerotic artery, (2) the transformation of a diseased artery to a healthier artery through the removal of lipids from the lesion, (3) an increase in the artery’s luminal diameter that allows an increase in blood flow, and (4) the return of the artery to a state that results in functional improvement. Plaque rupture appears to be responsible for the majority of vasculature-related clinical events. The stability of atherosclerotic lesions may be dependent on a variety of conditions, such as the presence, nature, and location in the lesion of lipid-rich regions; inflammatory cells; a fibrous cap; calcified areas; etc. As such, some or all of the changes described above may increase the stability of the intimal lesions and reduce the likelihood of plaque rupture and a clinical event.

In the current study, plaque size in the abdominal aorta did not decrease during the regression phase of the trial, despite the biochemical changes in lipids that reflected plaque regression. As previously reported, coronary artery plaque size also did not decrease with plasma lipid lowering in these animals.17 Cholesterol content in the abdominal aorta was reduced in animals consuming a lipid-lowering diet for 30 months compared with the baseline group, suggesting an efflux of lipid (to \( \approx 40\% \) of baseline levels) from the artery during the regression phase. The regression-induced decreases in aortic cholesterol were not affected by the CEE or CEE+MPA therapies. The aortic cholesterol levels of the regression groups (5 to 6 \( \mu g/mg \) wet weight) remained higher than in normal aortas (1 to 2 \( \mu g/mg \) wet weight) on the basis of previous studies.5,6,21 Malinow22 obtained similar findings in female cynomolgus macaques fed an atherogenic diet for 6

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Total cholesterol content of the abdominal aorta. Results are expressed as micrograms of cholesterol per milligram LFDW.
months and then a plasma lipid-lowering diet for 18 months. Aortic cholesterol content was lower in regression groups than in baseline animals but remained higher than that of normal aortas.

In a previous study of atherosclerosis regression in male rhesus monkeys, animals with 19 months of diet-induced hyperlipidemia (TPC concentrations of ≈800 mg/dL) underwent dietary lipid lowering (TPC concentrations of 180 to 220 mg/dL) for 24 or 48 months, resulting in an abnormally aortic cholesterol concentration of ≈2 to 3 mg/g wet aorta compared with baseline levels of ≈10 mg/g wet aorta. These levels were only slightly higher than the cholesterol content of the abdominal aorta (≈1.5 mg/g wet aorta) from nonatherosclerotic animals. The atherogenic and regression diets in that rhesus study contained 40% of calories as fat (lard) and differed only in the dietary cholesterol content in the regression phase, which was modified to achieve TPC concentrations in the 200 or 300 mg/dL range. The abdominal aortas of the group with TPC concentrations in the 300 mg/dL range contained ≈6 μg cholesterol per milligram wet weight, which is comparable to that observed in the current study. However, the regression group with TPC concentrations in the 200 mg/dL range (comparable to the TPC concentrations of 170 to 180 mg/dL in the regression phase of the current study) achieved a more complete return of aortic cholesterol toward normal levels than found in the current study, despite the higher overall dietary fat content (40% of calories versus 30%) during the regression phase. Differences between the two studies that might account for this result include (1) a longer induction phase in the current study (24 months versus 19 months), (2) differences between induction and regression diets other than fat calories (eg, the inclusion of butter in the progression phase of the current study), (3) differences in atherosclerosis (aortic cholesterol content) at baseline that were slightly greater in the current study (≈14 mg cholesterol per gram of wet aorta versus 11 mg per gram of wet aorta in the rhesus study), (4) differences between males and females, (5) species differences between rhesus and cynomolgus monkeys, or (6) other unknown factors.

The continued presence of elevated cholesterol concentrations in the abdominal aorta may have resulted from the moderate lipid-lowering diet, which is comparable to that recommended by the American Heart Association (30% of calories as fat and the equivalent of 100 mg cholesterol/d per person). It is possible that a more aggressive approach with respect to dietary fat and cholesterol or a longer exposure to the lipid-lowering diet might have resulted in continued loss of the cholesterol from the artery. It is unclear which effects estrogen replacement therapy or hormone replacement therapy would have on lipid or connective tissue changes under such circumstances.

The preferential reduction of cholesterol ester over free cholesterol in the regression groups is consistent with that observed in previous studies. The lack of an effect of estrogen replacement on aortic cholesterol may reflect a relatively minor influence of estrogen on regression compared with the large effect of the lipid-lowering diet. It is currently unknown whether estrogen plays a role in arterial cholesterol efflux, although some investigators have suggested that arterial lipoprotein uptake and degradation may be influenced by estrogen.

Connective tissue content of the abdominal aorta was affected by both regression and treatment. Collagen content (as estimated by hydroxyproline content per LFDW) was higher in the control and CEE+MPA groups than in the baseline animals, with intermediate levels in the CEE-only treatment group. Abdominal aortic elastin content was lower in the control and CEE+MPA groups than in the baseline group, again with the CEE-only group having intermediate levels. Aortic collagen and elastin contents of the CEE group were not different from those of the baseline, control, or CEE+MPA groups when the 4 study groups were analyzed together. However, restriction of the analysis to the 3 regression groups demonstrated that CEE aortic collagen was lower than that of the control group and tended to be lower than that of the CEE+MPA group. Taken together, the results demonstrate that CEE inhibited collagen accumulation associated with atherosclerosis regression and suggest that MPA may antagonize that effect.

Interestingly, several recent studies have reported that MPA attenuated or reversed the beneficial effects of CEE on a number of vascular end points or cardiovascular risk factors. In 1 report, the development of diet-induced coronary artery atherosclerosis in ovariectomized animals was inhibited by CEE therapy, whereas the addition of MPA to the CEE regimen completely abolished the CEE effect. Other studies have shown that the beneficial effect of CEE on coronary artery vasomotor reactivity was attenuated by MPA given either cyclically or continuously. Nevertheless, the reversal of the atheroprotective effect of CEE by MPA may not apply to all progestins, as cyclic progesterone implants had no negative impact on the protective effect of continuous...
Hormone Effects on Atherosclerosis Regression

17β-estradiol implants against diet-induced coronary artery atherosclerosis in a previous study. These discrepancies in hormone effects on the cardiovascular system are important and could be related to the type of estrogen, the type of progestin, or the frequency or route of administration, demonstrating the need for more work in this area.

The prevention of aortic collagen accumulation in the CEE group may have been mediated through direct effects of estrogens on arterial cell metabolism. Collagen synthesis has been shown to be inhibited by 17β-estradiol in cultured aortic smooth muscle cells, whereas no effect on cell proliferation was observed. This effect may be regulated through specific receptors for estrogens, since aortic smooth muscle cells have been shown to express mRNA for both the classic estrogen receptor (ERα) and the newly described ERβ. Specific receptor-mediated mechanisms by which estrogen may control collagen metabolism remain to be determined, especially since recent studies suggest that ligand-specific effects may be regulated differently through ERα or ERβ, depending on the composition of the regulatory regions of individual genes.

The expression of results per unit artery area reduces the impact of changes in arterial composition and mass that occur during atherosclerosis progression and regression. Lesion progression is accompanied by increases in the numbers of macrophages, smooth muscle cells, and other cells in the intima, along with alterations in the lesion contents. Progression is accompanied by efflux of lipid and other modifications. When expressed on an area basis, the elastin content was lower in the control and CEE+MPA groups than in the baseline animals, suggesting that loss of elastin occurred during regression in these animals. Although the collagen content per unit area was not statistically different between groups, regression groups tended to have higher amounts of collagen per unit area than did the baseline group. Given these changes, the abdominal aortas from the control and CEE+MPA groups would be expected to be less elastic or compliant.

Armstrong and Megan studied atherosclerosis regression in male cynomolgus macaques fed an atherogenic diet (1.2% cholesterol) for 17 months followed by a low-fat, cholesterol-free regression diet for up to 20 months. Collagen concentration (milligrams of collagen per gram LFDW) increased as a result of diet-induced atherosclerosis, with 50% to 75% increases in the more elastic arteries (eg, aorta, common carotid, and subclavian) and 33% increases in the more muscular arteries (eg, coronary and femoral) compared with nonatherosclerotic controls. Collagen concentrations of the aortas obtained from the group undergoing 7 months of plasma lipid lowering were ~10% higher than those of aortas from the atherosclerotic baseline group. Elastin, expressed per unit arterial weight or length, was highest in the baseline group and lower in the regression groups. Their findings are comparable to those of the present study. However, expression of collagen and elastin per unit of artery length gave a slightly different result; aortic collagen content was slightly lower in the 20-month regression groups than in baseline, suggesting that collagen also was being lost from the tissue. That finding contrasts with results from the current study using male rhesus monkeys and in previous studies using male rhesus monkeys on a moderate regression diet, in which aortic collagen increased in the regression groups. It is possible that regression induced by the low-fat, no-cholesterol diets in the study of Armstrong and Megan may have facilitated the reduction of the absolute amounts of collagen from the aorta, as opposed to a diet with moderate amounts of fat and cholesterol, such as that in the present study and in previous studies.

Histomorphometric analysis of a midsection of the abdominal aorta showed that the control group had increased artery size (ie, IELA) and a trend toward increased lumen size than did the baseline group, suggesting that remodeling of the artery to a larger size had occurred. Unfortunately, the size of the abdominal aorta can only be estimated in our report, because the sections were not perfusion fixed under pressure. Lumen stenosis of the coronary arteries was lower than baseline in the regression groups in the reports by Malinow, although this effect may have been the result of artery remodeling and not due to reductions in actual plaque area. Zarins et al examined the relationship between the regression of diet-induced atherosclerosis and enlargement of the aorta in male cynomolgus monkeys. They found a reduced abdominal aortic medial thickness, along with a preferential enlargement of the abdominal aorta (shown histologically by lumen area and by increased area encompassed by internal elastic lamina), in 6 animals fed a progression diet for 6 months followed by a regression diet for 6 months, compared with a baseline group (n=6) necropsied after 6 months of progression. The loss of aortic elastin during regression seen in the current and previous studies could be a factor in subsequent aortic enlargement and may have important implications for the development of AAAs, especially after regression of atherosclerosis. AAA rupture is a significant cause of mortality in the elderly and an important health problem; up to 14% of the male population >65 has measurable aneurysmal development. The incidence of AAA in the female population >65 is ~6%, because AAA development in women appears to lag ~10 years behind that of men, perhaps due to the protective effects of estrogen over their lifetime. Nevertheless, the incidence of AAA in both sexes increases with age, and thus, the clinical significance of AAA will also increase as the population ages.

Our results demonstrate that abdominal aortas of surgically menopausal female cynomolgus monkeys with diet-induced atherosclerosis undergo chemical remodeling in the wake of a lipid-lowering diet patterned after that recommended by the American Heart Association. In addition, detrimental connective tissue changes generally associated with lesion regression (ie, the accumulation of collagen and the loss of elastin) may be inhibited by CEE treatment. These beneficial effects may be antagonized by MPA. Although plaque area did not decrease as a result of plasma lipid lowering, the reduction in cholesterol and cholesterol ester in the lesions may lead to more stable plaques, reducing the potential for plaque rupture and therefore providing a clinical benefit. An additional benefit of plasma lipid lowering may relate to improvement in vascular tone, since the paradoxical constriction to acetylcholine infusion commonly seen in atherosclerotic animals...
during the progression phase was not seen in the regression groups in this study. This improvement in vascular tone may also decrease the likelihood of plaque rupture.

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Conjugated Equine Estrogens Alone, but Not in Combination With Medroxyprogesterone Acetate, Inhibit Aortic Connective Tissue Remodeling After Plasma Lipid Lowering in Female Monkeys

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