Inhibition of Coronary Artery Atherosclerosis by 17-beta Estradiol in Ovariectomized Monkeys
Lack of an Effect of Added Progesterone

Michael R. Adams, Jay R. Kaplan, Stephen B. Manuck, Donald R. Koritnik, John S. Parks, Mary S. Wolfe, and Thomas B. Clarkson

Although controversy continues, the preponderance of evidence indicates that estrogen replacement therapy favorably influences the risk of coronary heart disease in postmenopausal women. It remains uncertain how this effect is mediated and whether the cyclic addition of a progestin may influence adversely an estrogen-related cardioprotective effect. We investigated the influence of sex hormone replacement therapy on diet-induced coronary artery atherosclerosis in estrogen-deficient (ovariectomized) adult female cynomolgus monkeys. Monkeys were assigned randomly to one of three treatment groups: 1) no hormone replacement (n=17), 2) continuously administered 17-beta estradiol plus cyclically administered progesterone (n=20), and 3) continuously administered 17-beta estradiol (n=18). The physiologic patterns of plasma estradiol and progesterone concentrations were maintained by administering the hormones in sustained-release subcutaneous Silastic implants. The experiment lasted 30 months. At necropsy, coronary artery atherosclerosis was inhibited similarly (reduced by approximately one-half) in animals in both hormone replacement groups (p<0.05). Antiatherogenic effects of hormone replacement were independent of variation in total plasma cholesterol, lipoprotein cholesterol, apoprotein A-1 and B concentrations, high density lipoprotein subtraction heterogeneity, and low density lipoprotein molecular weight. We conclude that physiologic estrogen replacement therapy with or without added progesterone inhibits atherosclerosis progression in ovariectomized monkeys. This may explain why estrogen replacement therapy results in reduced risk of coronary heart disease in postmenopausal women.

Premenopausal white women are at a low risk of coronary heart disease relative to age-matched white men. Although direct evidence is lacking, it is widely believed that ovarian estrogen is responsible for this gender-related protection. However, there is no direct evidence that endogenous estrogen or progesterone influences the pathogenesis of coronary heart disease or its underlying cause, coronary artery atherosclerosis. Furthermore, it remains uncertain whether coronary risk in women is influenced by physiologic conditions that affect endogenous sex steroid levels. While most studies have found evidence for increased severity of coronary artery atherosclerosis and increased coronary risk in postmenopausal women, others have found no relationship. The results of studies of the effects of pregnancy, a hyperestrogenic and hyperprogestogenic state, are equally divided; approximately half the studies indicate that coronary risk increases with increasing number of pregnancies and half find no relationship.

As regards exogenous estrogen, the preponderance of evidence indicates that estrogen replacement therapy favorably influences coronary risk in postmenopausal women. More controversial is whether the cyclic addition of a progestin to an estrogen replacement regimen may adversely influence an estrogen-related protective effect. This concern has arisen because of evidence that some progestins may neutralize the favorable effect of estrogen replacement therapy on plasma lipoprotein patterns.

We have utilized a nonhuman primate model of atherosclerosis, the cynomolgus macaque (Macaca fascicularis) fed an atherogenic diet, to address questions regarding the effects of gender and sex hormones on the development of coronary artery atherosclerosis. We have previously shown that, as in white human beings in most Western societies, there is a marked gender difference in the extent of coronary artery atherosclerosis: female monkeys are much less affected than are males. We also have shown that ovariectomy results in an approximate doubling of the extent of atherosclerosis, while repeated pregnancy results in an approximate 50% reduction in the extent of atherosclerosis. Furthermore, the pregnancy-related inhibition of atherosclerosis is associated with the persistently elevated concentration of circu-
Animals was reduced to the original amount (Table 1). The monkeys to diet-induced coronary artery atherosclerosis 7 and its than expected; thus, at month 26, the cholesterol content 

Experimental Design and Procedures

Methods

Animals

The study animals were 62 female cynomolgus macaques imported from Indonesia as adults. This species was selected because of its well-known susceptibility to diet-induced coronary artery atherosclerosis9 and its previous usefulness in studies of reproductive influences on coronary artery atherosclerosis.4,5,6

Table 1. Diet Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, USP</td>
<td>8.00</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>8.00</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>35.44</td>
</tr>
<tr>
<td>Dextrin</td>
<td>6.00</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5.00</td>
</tr>
<tr>
<td>Applesauce</td>
<td>4.50</td>
</tr>
<tr>
<td>Lard</td>
<td>9.50</td>
</tr>
<tr>
<td>Butter</td>
<td>3.00</td>
</tr>
<tr>
<td>Beef tallow</td>
<td>7.00</td>
</tr>
<tr>
<td>Dry egg yolk</td>
<td>3.50</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>0.50</td>
</tr>
<tr>
<td>Complete vitamin mixture</td>
<td>2.56</td>
</tr>
<tr>
<td>Ausman-Hayes mineral mixture</td>
<td>5.00</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>2.00</td>
</tr>
</tbody>
</table>

In this diet, 40% of calories were derived from fat, 0.25 mg cholesterol/calorie.

Hormone Manipulations

After consuming the atherogenic diet for a 4-month pretreatment period, all animals were ovarioctomized and, with a stratified randomization design, were assigned randomly to one of three experimental groups so that the groups were well-matched in regard to pretreatment total plasma cholesterol (TPC), plasma high density lipoprotein cholesterol (HDLC), and age. There were three experimental groups: untreated controls (n=18), estrogen and progesterone replacement (n=20), and estrogen replacement (n=17).

To mimic the physiologic concentrations of circulating estrogen and progesterone,6 sex hormones were administered by subcutaneous Silastic implant (Dow Corning, Midland, MI). Beginning 1 month after ovarioectomy, the animals in both hormone-replacement groups (2 and 3) were implanted subcutaneously with Silastic tubing (0.335 cm inner diameter, 0.465 cm outer diameter) which was 3.0 cm in length and filled with crystalline 17-beta estradiol (Steraloids, Inc., Wilton, NH). These implants remained in place for the entire 25-month treatment period. The animals in group 2 (estrogen and progesterone) were implanted subcutaneously with identical Silastic tubing, which was 3.5 cm in length and filled with crystalline progesterone (Steraloids, Inc.). Progesterone was administered only during alternating 28-day periods (i.e., every 28 days implants were either inserted or removed). To determine the continued effectiveness of delivery of the hormones, plasma estradiol6 and progesterone10 concentrations were determined at 3- to 4-month intervals. Untreated controls were implanted subcutaneously with empty Silastic tubing, which was 3 cm in length and remained in place continuously for 25 months.

Risk Variables

During the 4-month pretreatment period and a 25-month treatment period, determinations were made of several known or suspected atherosclerosis risk variables.

1. TPC11 and plasma HDLC12 concentrations were determined at 2- to 3-month intervals.
2. At months 4, 8, and 16, plasma lipoprotein patterns were assessed. Lipoprotein fractions were separated by ultracentrifugation and agarose column chromatography,13 and the cholesterol content of each fraction was quantitated.14 In macaques, four major fractions are ob-
tained. In addition to very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL, a fourth peak, termed intermediate density lipoprotein (IDL), exists. IDL is intermediate in molecular weight to VLDL and LDL. By using this procedure, LDL size (molecular weight) was also determined for each sample by including a trace amount of iodinated LDL of known molecular weight.15

3. Prepoured polyacrylamide gradient gels (4% to 30%, Pharmacia, Piscataway, NJ) were used at months 4, 8, and 16 to assess HDL subfraction size heterogeneity.16

4. Plasma concentrations of apoprotein A-1 (apo A-1) and apoprotein B (apo B) were determined at months 4, 8, and 16 by using enzyme-linked immunosorbent assays.17,18

5. Blood pressure was determined at 6-month intervals.19

6. Fasting blood glucose20 and insulin21 and the glucose and insulin responses to intravenous glucose challenge21 were determined at yearly intervals.

7. Plasma estradiol9 (measured at 3-month intervals) was assessed as a predictor of atherosclerosis extent. Also, the binding capacities of sex hormone-binding globulin (SHBG)22 and corticosteroid-binding globulin (CBG)22 were determined to assess the potential associations with atherosclerosis extent.

8. Social status, or dominance, refers to the relative abilities of individuals within social groups to defeat other group members in agonistic or competitive encounters. We have previously shown that low social status is a significant predictor of atherosclerosis extent in female monkeys.5-23 As in previous experiments, social status was determined weekly on the basis of the outcomes of aggressive interactions.5,23 The animal in each group that defeated all others was designated the first ranking monkey. The monkey that defeated all but the first ranking animal was designated second ranking, and so forth. The number of animals over which a given monkey was dominant was recorded as a dominance score for that animal. Animals falling above the median of the distribution of social rankings were identified as dominant monkeys and the remaining, lesser ranked animals were designated subordinate monkeys. Importantly, social rankings were stable over the course of the experiment.

Necropsy and Measurement of Atherosclerosis

At the time of necropsy, the animals were anesthetized deeply with pentobarbital. Each cardiovascular system was flushed with normal saline and perfused with 10% neutral buffered formalin under a pressure of 100 mm Hg. The carotid arteries and the aortas were opened longitudinally. Each cardiovascular system was opened and the aortas were opened longitudinally and were immersion-fixed in 10% buffered formalin. Five equally spaced cross-sections were taken from the thoracic and abdominal aortas, and the plaque areas were determined by use of the digitizer. The extent of aortic atherosclerosis was expressed as the mean intimal area of the five sections. At the carotid bifurcation, one standard cross-section was taken for microscopic evaluation. Three serial sections were taken from each common carotid and iliac-femoral artery, and the mean intimal area was determined for each by using the digitizer.

Statistical Analysis

The atherosclerosis data were not normally distributed. Square root transformations of these data were used in all analyses to reduce skewness and to equalize group variances. Analysis of variance (ANOVA), repeated-measures ANOVA, analysis of covariance (ANCOVA), multiple regression, and Pearson's product-moment correlation were used for the statistical analyses. Duncan's new multiple-range test was used for post hoc comparisons. Statistical analyses were carried out with the BMDP statistical software package (University of California, Los Angeles, CA, 1985).

Results

Coronary Artery Atherosclerosis, Total Plasma Cholesterol, and Plasma High Density Lipoprotein Cholesterol

The mean intimal areas for the three treatment groups are presented in Table 2. The ANOVA revealed a significant main effect of the experimental condition ($F_{2,23}=3.16$, $p<0.05$). Post hoc comparisons among group means (by Duncan's multiple-comparison procedure, at $p<0.05$) revealed that coronary artery atherosclerosis in the untreated control (estrogen-deficient) monkeys was significantly more extensive than in animals in the two hormone replacement groups, and that there was no significant difference between the two hormone replacement groups. The TPC and HDLC concentrations are also summarized in Table 2. These values represent the means of all values for each animal during the 30-month experiment. While hormone replacement had no effect on mean TPC ($F_{2,23}=0.25$, $p<0.77$) and HDLC ($F_{2,23}=0.02$, $p>0.98$) (repeated measures ANOVA), or on TPC and HDLC at any time point, there were effects of treatment on temporal patterns, as indicated by a significant experimental group-by-time interaction term for both TPC ($F_{14,363}=2.28$, $p<0.006$) and plasma HDLC ($F_{14,363}=2.31$, $p<0.005$). This interaction appeared to be accounted for by a slight decrease in TPC and increase in HDLC among group 3 (estrogen only) animals at month 16 post-diet followed by a slightly exaggerated hypercholesterolemic response to the increase in dietary cholesterol at month 18. A significant main effect of time on TPC ($F_{14,363}=82.09$, $p<0.0001$) and HDLC ($F_{14,363}=79.11$, $p<0.0001$) was due to the increase in cholesterol content of the diet at month 18.

Atherosclerosis at Other Arterial Sites

Hormone replacement was associated with smaller intimal areas at all arterial sites examined (Table 3). However, these differences were statistically significant only in the iliac-femoral artery.
Table 2. Coronary Artery Atherosclerosis (Plaque Area), Total Plasma Cholesterol, and Plasma HDL Cholesterol Concentrations in Ovariectomized Monkeys

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n=17)</th>
<th>Group 2 (n=20)</th>
<th>Group 3 (n=18)</th>
<th>Main effect of experimental condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque area (mm²) (unadjusted)</td>
<td>0.227</td>
<td>0.101</td>
<td>0.099</td>
<td>F=3.16*</td>
</tr>
<tr>
<td>Plaque area (mm²) (adjusted)</td>
<td>0.204</td>
<td>0.105</td>
<td>0.109</td>
<td>p=0.05</td>
</tr>
<tr>
<td>Total plasma cholesterol*</td>
<td>463±26</td>
<td>433±30</td>
<td>448±33</td>
<td>F=7.41*</td>
</tr>
<tr>
<td>Plasma HDL cholesterol*</td>
<td>34.9±4.0</td>
<td>35.2±3.3</td>
<td>35.9±3.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Data analyzed after square root transformation. †Values are means derived by retransforming means of the transformed data. Adjustment was made by using ANCOVA with total plasma cholesterol, very low density lipoprotein cholesterol, HDL cholesterol, and apoprotein A-1 as covariates. $Means±SEM.

Table 3. Extent of Atherosclerosis in Aorta, Carotid, and Iliaco-femoral Arteries

<table>
<thead>
<tr>
<th>Site</th>
<th>Group 1 (n=17)</th>
<th>Group 2 (n=20)</th>
<th>Group 3 (n=18)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic aorta</td>
<td>1.342±0.186</td>
<td>0.891±0.136</td>
<td>1.037±0.225</td>
<td>NS</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>0.819±0.146</td>
<td>0.525±0.092</td>
<td>0.619±0.191</td>
<td>NS</td>
</tr>
<tr>
<td>Right common carotid</td>
<td>0.438±0.058</td>
<td>0.311±0.057</td>
<td>0.260±0.061</td>
<td>NS</td>
</tr>
<tr>
<td>Left common carotid</td>
<td>0.366±0.070</td>
<td>0.276±0.056</td>
<td>0.235±0.062</td>
<td>NS</td>
</tr>
<tr>
<td>Right carotid bifurcation</td>
<td>1.451±0.170</td>
<td>1.110±0.174</td>
<td>1.066±0.156</td>
<td>NS</td>
</tr>
<tr>
<td>Left carotid bifurcation</td>
<td>1.510±0.209</td>
<td>1.084±0.151</td>
<td>1.395±0.181</td>
<td>NS</td>
</tr>
<tr>
<td>Right iliaco-femoral</td>
<td>0.237±0.039</td>
<td>0.153±0.037</td>
<td>0.117±0.038</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Left iliaco-femoral</td>
<td>0.235±0.043</td>
<td>0.142±0.035</td>
<td>0.133±0.046</td>
<td>&lt;0.09</td>
</tr>
</tbody>
</table>

Values are plaque area in mm²±SEM.
NS=not significant.

Plasma Lipoprotein Patterns

There were no effects of treatment on the cholesterol content of VLDL, IDL, LDL, or HDL at any of the times these determinations were made. Similarly, there was no effect on average LDL size (molecular weight) or HDL subfraction heterogeneity.

Plasma apo A-1 and apo B data are summarized in Figures 1 and 2. There were suggestive between-group differences at baseline in plasma apo A-1 ($F_{2,52}=2.43, p=0.098$) and plasma apo B ($F_{2,52}=1.79, p=0.177$) concentration. Therefore, ANCOVA was used to determine the effects of treatment adjusted for these baseline differences. The combined estrogen and progesterone treatment resulted in a decrease in apo A-1 relative to the other two groups at month 8 ($F_{2,52}=7.85, p=0.001$) and month 16 ($F_{2,52}=3.52, p=0.038$) post-diet. The decrease in plasma apo A-1 seen in all groups at month 8 and the increase at month 16 correspond with the typical temporal responses of plasma HDL to increased dietary cholesterol. There was a suggestion of an effect of estrogen treatment on plasma apo B concentration at month 8 ($F_{2,52}=2.64, p=0.081$) but not at month 16 ($F_{2,52}=0.70, p=0.502$). Plasma apo B was generally increased at month 8 and decreased at month 16, corresponding to typical temporal responses of plasma LDL to increased dietary cholesterol.

Plasma Lipids and Lipoproteins as Predictors of Coronary Artery Atherosclerosis Extent

Multiple regression was used to determine which plasma lipid and lipoprotein variables were significant predictors of atherosclerosis extent. TPC, plasma HDLc, VLDL cholesterol, and apo A-1 were significant predictors and together accounted for 77% of the variability in extent of coronary artery atherosclerosis. TPC alone accounted for 67% of variability. Higher TPC and VLDL cholesterol and lower HDLc and apo A-1 predicted more extensive atherosclerosis. These variables were used as covariates in an ANCOVA to determine the effects of hormone replacement on coronary artery atherosclerosis, adjusted for significant lipoprotein risk factors. Adjusted values for each group are given in Table 2. Adjustment did not affect the relationships among the three groups. A significant main effect of the experimental group (treatment group) ($F_{2,52}=7.41, p<0.002$) persisted. Thus, while plasma lipid and lipoprotein variables accounted for more than three-fourths of the variability in atherosclerosis extent, the effects of hormone replacement remain unexplained by plasma lipoprotein variables measured in this experiment.

Other Risk Variables

Risk variables not affected by treatment were blood pressure, fasting blood glucose, carbohydrate tolerance,
Plasma apo A-1 (apo A-1) concentrations at baseline, month 8, and month 16 in untreated controls (filled bars), animals treated with estrogen and progesterone (lined bars), and animals treated with estrogen alone (open bars). Plasma apo A-1 was lower in untreated controls at baseline (p=0.098), and, when adjusted for baseline values, lower at month 8 (p=0.001) and month 16 (p=0.038) in monkeys treated with estrogen and progesterone. Across groups, values were generally lower at month 8 and higher at month 16, as expected, in response to diet.

Plasma apo B (apo B) concentrations at baseline, month 8, and month 16 in untreated controls (filled bars), animals treated with estrogen and progesterone (lined bars), and animals treated with estrogen alone (open bars). There were no differences at baseline (p=0.177) or month 16 (p=0.502). Differences at month 8 were of marginal statistical significance (p=0.081) when adjusted for baseline values.

Discussion

The cynomolgus macaque has been a useful model in atherosclerosis research for many years, due principally to its susceptibility to diet-induced atherosclerosis of the epicardial coronary arteries. It has been particularly useful in elucidating the effects of sex hormones on atherogenesis, as the reproductive physiology of this species is similar to that of human beings. Also, we have previously shown that, as with human beings in Western societies, there is a gender difference in the development of coronary artery atherosclerosis. Furthermore, there is an association between altered endogenous sex hormone patterns and the development of coronary atherosclerosis. In pregnancy, a hyperestrogenic state, atherosclerosis is inhibited. In ovarian deficiency, i.e., naturally occurring anovulation or surgical removal of ovaries, atherosclerosis is accelerated. The current experiment extends these findings by directly implicating the physiologic concentrations of exogenously administered natural estrogen in the inhibition of the development of atherosclerosis and by indicating that progesterone in physiologic concentrations, when combined with estrogen, does not modulate this effect.

It remains unknown whether similar effects occur in human beings; thus, great caution must be exercised in so extending these conclusions. However, this finding is potentially important because of the current controversy regarding the adverse effects of some progestins on plasma lipoprotein patterns. While evidence exists that an increasing dose of contraceptive progestins is associated with increased risk of coronary heart disease, there is no evidence that progestins and the progestin doses used in postmenopausal estrogen replacement therapy influence coronary heart disease risk. Also, there is no evidence that progestins adversely influence the progression of coronary artery atherosclerosis. To the contrary, previous findings from our laboratory have indicated that, despite causing substantial decreases in plasma HDL, contraceptive progestins do not adversely influence atherogenesis in monkeys if a sufficiently potent estrogen is co-administered. Also, among animals at high risk due to adverse plasma lipoprotein patterns, the atherosclerosis extent is actually decreased by oral contraceptive treatment, probably due to an atherosclerosis-inhibiting effect of the potent contraceptive estrogen, ethinyl estradiol. This effect was independent of contraceptive progestin-induced changes in plasma lipopro-
teins, although, within treatment groups, TPC and HDLC were predictive of atherosclerosis extent. The evidence from the current experiment indicates that parenteral physiologic replacement of the natural estrogen, 17-beta estradiol, also inhibits atherogenesis and that it does so independently of variation in plasma lipoprotein patterns. Physiologic progesterone replacement did not significantly modulate this response. It is not surprising that, in contrast to the effects of orally administered estrogens and progestins, the effects of these relatively mild estrogenic and progestogenic stimuli on lipoprotein metabolism were subtle or nonexistent. In the case of TPC and HDLC, the trends toward favorable effects of estrogen that were observed early in the study were neutralized across time by an apparent interaction with the influence of increased dietary cholesterol consumption. There were no apparent effects of treatment on plasma lipoprotein cholesterol concentrations, LDL molecular weight, HDL subfraction distributions, or plasma apo B concentrations, while the combined estrogen plus progesterone treatment slightly reduced plasma apo A-1 concentration. However, statistical adjustment for apo A-1 and other lipoprotein variables found to be associated with atherosclerosis extent did not alter, and in fact strengthened, the conclusion that estrogen replacement inhibits, and added progesterone has no modulatory influence on, atherogenesis. This does not, however, rule out the possibility that the effects of estrogen on aspects of lipoprotein metabolism or plasma lipoprotein patterns that were not assessed in this experiment may have played a role.

The effects on coronary artery atherosclerosis could not be explained by variation in other risk variables. In previous experiments, low social status has been associated with an increased extent of coronary artery atherosclerosis in female monkeys with intact ovaries, but not in ovarioctomized monkeys. In these experiments, evidence indicated that low social status was associated with relatively poor ovarian function and that the effects of low social status on atherogenesis were likely mediated by this relative estrogen deficiency. Therefore, in the current experiment, the lack of an association between atherosclerosis extent and social status is not surprising since, within groups, animals were similar in regard to ovarian function, (i.e., they had none) and in plasma sex steroid concentrations. This observation also strengthens the case for the effects of social status observed in previous experiments being mediated through effects on ovarian function.

Other possible explanations of the anti-atherogenic effects of estrogen involve the direct effects on atherogenesis at the level of the arterial intima. Estrogen and progestin receptors have been identified in arterial endothelial and smooth muscle cells of several mammalian species, most recently in the coronary arteries of human beings and cynomolgus monkeys (unpublished data). A recent study has shown that treatment of ovarioctomized baboons with 17-beta estradiol results in a redistribution of aortic intracellular estrogen receptors from the cytoplasmic fraction to the nuclear fraction and an increase in the cytoplasmic concentration of progesterone receptors. These findings imply a role for sex steroids in the regulation of arterial cell function. Other studies with animals have shown that estrogen treatment results in reductions in lipoprotein-induced arterial smooth muscle cell proliferation, inhibition of myointimal proliferation associated with mechanical endothelial injury, reduced cholesterol ester influx and hydrolysis, inhibition of platelet aggregation, increased prostacyclin production, and decreased collagen and elastin production by arterial smooth muscle cells. These findings provide further evidence that vascular estrogen receptors are physiologically functional and that estrogen may directly influence the atherosclerotic process.

Regardless of the mechanism, we conclude that estrogen deficiency results in an exacerbation of coronary artery atherosclerosis and, furthermore, that physiologic estrogen replacement in estrogen-deficient individuals inhibits, while added progesterone does not further affect, progression of the atherosclerosis. These findings may explain why estrogen replacement therapy results in a decreased risk of coronary heart disease in postmenopausal individuals.

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