Determinants of Coronary Artery Reactivity in Premenopausal Female Cynomolgus Monkeys With Diet-Induced Atherosclerosis

J. Koudy Williams, DVM; Carol A. Shively, PhD; Thomas B. Clarkson, DVM

Background The purpose of this study was to identify determinants of coronary artery reactivity among premenopausal female monkeys. Estrogen replacement therapy in postmenopausal females modulates reactivity of atherosclerotic coronary arteries. However, no studies have evaluated the factors that modulate coronary artery reactivity among premenopausal females.

Methods and Results Twenty-five adult premenopausal female monkeys were fed an atherogenic diet for 32 months. During this time, monkeys were housed in small social groups and determined to be socially dominant (associated with normal ovarian function) or subordinate (associated with impaired ovarian function). After 32 months, coronary artery vasomotor responses to intracoronary acetylcholine, nitroglycerin, and serotonin were assessed by computer-assisted quantitative coronary angiography. Coronary arteries of dominant monkeys dilated (+9±2%), whereas those of subordinate monkeys constricted (−6±2%) in response to acetylcholine (P<.05). There was no effect of social status on vascular response to nitroglycerin or serotonin (P>.10). Vascular responses to acetylcholine were independent of social status effects on plasma lipids, blood pressure, and atherosclerosis extent. The correlation between acetylcholine responses and plasma estradiol concentration measured on the day of angiography was r=+.7 (P=.01). Furthermore, dilation occurred only if plasma estradiol concentrations were greater than 60 pg/mL.

Conclusions Psychosocial factors and endogenous estrogen production are important modulators of acetylcholine-mediated dilation of atherosclerotic coronary arteries among premenopausal female monkeys. (Circulation. 1994;90:983-987.)

Key Words • stress • vasodilation • atherosclerosis • sex steroids

Coronary heart disease (CHD) is the leading cause of death among women in the United States.1,2 Premenopausal women are at lower risk for developing CHD than white men of similar age.1,2 However, women are more likely than men to have angina rather than myocardial infarction.3 Some forms of angina pectoris in men and women are thought to be due to coronary vasospasm,4 and these forms of angina often appear in patients with angiographically normal coronary arteries.5,6 However, the pathogenesis of angina pectoris in women remains unclear.

It is believed that impaired reactivity of atherosclerotic coronary arteries may contribute to the pathogenesis of vasospasm and angina pectoris.5,7 Atherosclerotic arteries are known to have impaired dilator responses and augmented constrictor responses to a variety of neurohumoral stimuli.8 However, reactivity of arteries may be affected by factors other than atherosclerosis. For example, psychosocial stress impairs atherosclerotic coronary artery reactivity in male monkeys independent of atherosclerosis extent and plasma lipid concentrations.8 Furthermore, evidence is accumulating that ovarian hormones influence reactivity of atherosclerotic coronary arteries. Results of studies in surgically postmenopausal cynomolgus monkeys9,10 and postmenopausal women12 indicate that both long-term and short-term estrogen treatment prevent impaired acetylcholine-mediated dilation of atherosclerotic coronary arteries. However, no studies have examined the effects of impaired coronary artery reactivity in premenopausal females.

Cynomolgus monkeys living in social groups form social status hierarchies. Previous work with group-living female cynomolgus monkeys has shown that, compared with their subordinate counterparts, dominant animals have priority of access to resources such as food and water, which reduces stress; better ovarian function; lower plasma cholesterol concentrations; and less extensive diet-induced coronary artery atherosclerosis.13 Thus, social subordination in female monkeys is associated with certain risk factors for CHD. However, no studies have identified the modulators of coronary artery reactivity of premenopausal women or of female monkeys. In the study reported here, the determinants or modulators of coronary artery reactivity among premenopausal female monkeys with diet-induced atherosclerosis were examined. The results of this study identify potential factors that may contribute to the pathogenesis of angina in premenopausal women.

Methods

Subjects

Adult female cynomolgus monkeys (Macaca fascicularis, n=48) were used in this study. The animals were estimated to be 6 to 12 years of age on the basis of dentition. These females
were chosen from a pool of 72 adult female monkeys fed the experimental diet (described below) for 1 month before the experiment began. Total plasma cholesterol (TPC) and high-density lipoprotein cholesterol (HDL-C) concentrations were determined by the methods described below, and monkeys within the upper two thirds of the distribution of the TPC:HDL-C ratio were chosen for this study. Thus, the animals chosen represent a subset of the adult female population that is at increased risk of developing atherosclerosis. Six animals died from causes unrelated to the experiment (primarily diarreal diseases). Of the 42 remaining animals, a random sample of 25 monkeys was chosen for the vascular responsiveness studies. All procedures involving animals were conducted in compliance with state and federal laws, standards of the Department of Health and Human Services, and guidelines established by the institutional Animal Care and Use Committee and the American Physiological Society.

**Diet**

The monkeys were fed a semipurified diet ad libitum for 32 months. The diet contained 0.25 mg cholesterol/calorie and 40% of calories as fat. It was designed to approximate the amounts of cholesterol (188 mg cholesterol/d) and saturated fat (40% of calories) consumed by the average North American person.14

**Experimental Design**

The monkeys began consuming the atherogenic diet a month before the experiment to aid in subject selection (see above) and continued to consume the diet throughout the experiment. They were housed for 3 months in single cages, and preexperimental measures of TPC, HDL, and blood pressure (BP) were collected periodically. The monkeys then were assigned randomly to four-member social groups in which their social status stabilized and was determined. Monkeys remained in social groups throughout the rest of the experimental period.

The following determinations were made during the experiment:

**Plasma Cholesterol Concentrations**

Plasma TPC15 and HDL-C16 concentrations were measured every 4 months. The values reported are the average values for the last year of the experiment.

**Blood Pressure**

Systolic (SBP) and diastolic blood pressures (DBP) were measured every 4 months during the experiment by indirect methods.17 The values reported are the average values for the last year of the experiment.

**Menstrual Cycle Determinations**

Cynomolgus monkeys have a 28-day menstrual cycle similar to that of women. Their follicular and luteal phases are similar to those of women in their relative length within a cycle and the estradiol and progesterone concentrations during these phases. The monkeys were trained to present themselves for vaginal swabbing to detect menses and for venipuncture for blood sample collection three times per week throughout the experiment.

Plasma progesterone concentrations (ng/mL) were determined by radioimmunoassay (Diagnostic Products Corp). Luteal phase progesterone concentrations were used as an indicator of menstrual cycle quality. Progesterone concentrations greater than 4 ng/mL indicate that ovulation has occurred, whereas progesterone concentrations less than 2 ng/mL indicate an anovulatory cycle.13 The highest progesterone concentration that occurred during the luteal phase (the 2 weeks preceding menses) was chosen from each menstrual cycle. Peak progesterone concentrations were averaged for the last year of the experiment (mean peak progesterone). Additiously, progesterone and estradiol concentrations were determined by radioimmunoassay on the day of vascular reponsiveness studies (angiography).

**Social Status**

The social status of an individual refers to that animal’s ability to defeat other members of its social group in aggressive or competitive interactions. Social status was determined monthly throughout the experiment by recording the outcomes of aggressive interactions between cagemates. The animal in each social group that defeated all other group members was designated the first-ranking monkey. The animal that defeated all but the first-ranking monkey was designated the second-ranking monkey and so forth. The resulting social status hierarchies for each social group were stable over time. Animals that were first or second ranking, on average, were considered dominant (n=13), while third- or fourth-ranking animals were considered subordinate (n=12).

**Coronary Artery Reactivity**

Coronary artery reactivity was measured just before euthanasia and necropsy. Monkeys were anesthetized with ketamine hydrochloride (10 to 15 mg/kg body weight IM) and butorphanol (0.025 mg/kg IM). Periodic doses of both agents were given to maintain light anesthesia, and the animals were allowed to breathe spontaneously. A catheter was inserted into the right femoral artery and advanced to the midthoracic aorta for measurement of BP and heart rate. A custom-designed 3F (tapered to 1.8F) catheter was inserted into the left femoral artery and advanced to the left main coronary artery with fluoroscopic guidance. BP was monitored from the tip of the coronary catheter to exclude damping and significant obstruction of coronary blood flow.

With an infusion pump (Harvard Apparatus), serial 2.5-minute intracoronary infusions were made in the following sequence: (1) 5% dextrose in water (control); (2) acetylcholine $10^{-8}$ mol/L (estimated assuming left coronary blood flow of 10 mL/min); (3) nitroglycerin (40 µg/min); (4) control; and (5) serotonin (10$^{-6}$ mol/L). After each infusion, cineangiographic images were obtained in the 30th right anterior oblique projection at 60 frames/s. Images were taken during a hand injection of 2 mL of nonionic contrast solution (Omnipaque, Squibb) into the left main coronary artery. Approximately 10 minutes elapsed between drug infusions and 30 minutes between nitroglycerin and the second control. Because of the lasting effects of serotonin on arteries, the order of infusions was not randomized.

Quantitative angiography was done in the Bowman Gray Cardiology Image Analysis Laboratory. A single frame from the angiogram was selected for analysis on the basis of clarity of the image of the proximal 2 to 3 cm of the circumflex coronary artery. Criteria for clarity included maximal opacification, no overlapping structures, and minimal motion artifact. The frame was subsequently digitized using a Cipro cinefilm projector and a Digitron remote workstation (Siemens). Quantitative angiographic software (Gammaris) specifically modified for our laboratory was used to detect the edges of the vessel segment of interest and measure their average pixel diameter. The same segment of artery was analyzed after each of the five infusions. The entire process of frame selection and quantitative analysis was repeated four different times. The results of the four analyses were highly correlated (r=.93). The average of the results of the four analyses was used to calculate the percent change in pixel diameter associated with each infusion. This method has been validated previously for evaluation of coronary artery reactivity in monkeys.9,11

**Coronary Artery Atherosclerosis**

Monkeys were euthanized with sodium pentobarbital (80 mg/kg IV) after angiography. The cardiovascular system then was flushed with normal saline solution and perfused with 10%
neutral buffered formalin at a pressure of 100 mm Hg for 1 hour. The heart was removed and immersed in 10% neutral buffered formalin. Morphometric analysis of plaque size was obtained for the left circumflex coronary artery. Five serial tissue blocks were cut at approximately 3-mm intervals and perpendicular to the long axis of the artery. Histological sections were stained with Verhoef's and van Gieson's stains. These sections were projected, and the cross-sectional area of plaque lesion was measured with a digitizer. Atherosclerosis extent was expressed as the mean cross-sectional area of the intima in square millimeters.

Statistics
The effect of social status on experimental variables (plasma lipids, BP, sex hormones, plaque extent, and vascular reactivity) was assessed using Student's unpaired t tests. The Pearson product-moment correlation was used to determine if vascular reactivity was associated with other experimental variables. Values presented are mean±SEM. All probability values are the result of two-sided tests and were considered significant at the 5% confidence level.

Results

Physiological Characteristics of Socially Dominant and Subordinate Females
In the subset of animals used in this experiment, there were no differences between dominant and subordinate animals in TPC or HDL-C concentrations, SBP or DBP, or coronary artery atherosclerosis extent (measured as intimal area) (all P values <.05; Table 1). Mean peak luteal phase progesterone concentrations were higher in dominant females than in subordinates, indicating that dominant females overall had better ovarian function (Table 1). However, progesterone concentrations measured on the same day as angiography were not different (P>.05).

Reactivity of Coronary Arteries Among Dominant and Subordinate Monkeys
There was no effect of social status on vascular responses to nitroglycerin or serotonin (P>.05; Fig 1). However, coronary arteries of dominant animals dilated and those of subordinate animals constricted in response to acetylcholine (P<.05, Fig 1).

Relations Between Physiological Characteristics and Coronary Artery Reactivity
Coronary artery reactivity to nitroglycerin and serotonin was not significantly correlated with any other physiological characteristics (for all, P>.01). Correlations between acetylcholine responses and progesterone concentrations on the day of angiography, atherosclerosis extent, and plasma lipids were low (all r values <.25; Table 2). There was a modest correlation between acetylcholine response and mean peak progesterone (r=.48, P=.1; Table 2), indicating that quality of ovarian function was modestly associated with endothelium-mediated response. However, the closest association

### Table 1. Cardiovascular and Sex Hormone Parameters of Socially Dominant (n=13) and Subordinate (n=12) Premenopausal Cynomolgus Monkeys (mean±SEM)

<table>
<thead>
<tr>
<th>Social Status</th>
<th>Dominant</th>
<th>Subordinate</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC, mmol/L</td>
<td>8.15±1.09</td>
<td>7.76±0.70</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.55±0.29</td>
<td>1.89±0.18</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Atherosclerosis extent (intimal area, mm²)</td>
<td>0.2±0.06</td>
<td>0.25±0.1</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>103±4</td>
<td>103±6</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>61±4</td>
<td>62±5</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Same-day estrogen, pg/mL</td>
<td>130±32</td>
<td>56±12</td>
<td>&lt;.05*</td>
</tr>
<tr>
<td>Same-day progesterone, ng/mL</td>
<td>1.5±0.7</td>
<td>1.5±0.6</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>MPP, ng/mL</td>
<td>10±1.2</td>
<td>7.3±1</td>
<td>&lt;.05*</td>
</tr>
</tbody>
</table>

TPC indicates total plasma cholesterol; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; and MPP, mean peak progesterone.

*P<.05 between groups.

### Table 2. Correlations Between Vascular Responses and Other Experimental Variables in Premenopausal Cynomolgus Monkeys

<table>
<thead>
<tr>
<th>Substance Infused</th>
<th>Acute Estrogen</th>
<th>Acute Progesterone</th>
<th>MPP</th>
<th>Rank</th>
<th>IA</th>
<th>TPC</th>
<th>HDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>.7*</td>
<td>.01</td>
<td>.48†</td>
<td>.69*</td>
<td>-.13</td>
<td>.11</td>
<td>.21</td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td>.13</td>
<td>-.38</td>
<td>-.16</td>
<td>.23</td>
<td>-.1</td>
<td>-.12</td>
<td>-.1</td>
</tr>
<tr>
<td>Serotonin</td>
<td>-.01</td>
<td>.1</td>
<td>.02</td>
<td>-.1</td>
<td>.02</td>
<td>.18</td>
<td>-.31</td>
</tr>
</tbody>
</table>

MPP indicates mean peak progesterone; IA, intimal area; TPC, total plasma cholesterol; and HDL-C, high-density lipoprotein cholesterol.

*P<.05.
†P<.10.
Estrogen Production and Coronary Artery Reactivity

Studies of atherosclerotic monkeys and women indicate that physiological and supraphysiological doses of estrogen have beneficial effects on acetylcholine-mediated dilation of coronary arteries early in the progression of atherosclerosis.10-12 We have shown that intravenous administration of ethynyl estradiol, resulting in plasma estradiol concentrations of approximately 350 pg/mL, can change acetylcholine-induced constriction of coronary arteries to dilation within 20 minutes of administration to postmenopausal monkeys.11 Results of the present experiment extend those of previous studies by showing that endogenous estrogen production may modulate acetylcholine-mediated dilation of atherosclerotic coronary arteries and suggest a dose-dependent effect of plasma estradiol on coronary artery reactivity. There is a fairly strong relation between response to acetylcholine and plasma estradiol concentrations, with constriction changing to dilation at approximately 60 to 80 pg/mL estradiol (Fig 2). It is unclear, however, if this represents a linear relation or a threshold effect. The data in Fig 2 could be interpreted both ways, since the linear relation is suggested by three to four data points at very high doses of estradiol. The $r$ value drops to .4 when these data points are removed. Perhaps the vascular reactivity-estriadiol relationship is not as strong at lower estradiol concentrations, and a critical plasma concentration (ie, 80 pg/mL) is needed to affect vascular reactivity.

This study was not designed to examine definitively a dose-dependent or threshold hypothesis of estradiol's effects on coronary artery reactivity. This study does, however, provide a rough estimate of the plasma estradiol concentration needed to modulate coronary artery reactivity (60 to 80 pg/mL). This estradiol concentration is similar to that achieved in women taking postmenopausal estrogen replacement therapy and much lower than estradiol concentrations shown previously (300 to 400 pg/mL) to affect reactivity of atherosclerotic coronary arteries.10-12

Progesterone and Coronary Artery Reactivity

Social subordination in female monkeys has been associated with impaired ovarian function.13 In the present experiment, social status was inversely associated with mean peak progesterone concentrations. Others have shown that acute administration of progesterone improves reactivity of isolated rabbit coronary arteries.18 Therefore, it is conceivable that the higher progesterone concentrations we found in dominant monkeys may have a beneficial effect on coronary artery reactivity in vivo.

Potential Mechanisms

The two characteristics most closely associated with acetylcholine-mediated dilation were social status and plasma estradiol concentrations on the same day as angiography. This experiment was not designed to determine the mechanisms by which estradiol and psychosocial stress modulated acetylcholine-induced dilation of atherosclerotic coronary arteries. Acetylcholine activates muscarinic receptors in endothelial and smooth muscle cells; therefore, it cannot be determined from...
the present study whether estradiol and psychosocial factors affected the function of one or both of these vascular cell types. Psychosocial factors have been shown to affect reactivity, possibly through nitric oxide mechanisms. There are conflicting data as to whether estrogen affects reactivity of arteries through endothelium-mediated or endothelium-independent mechanisms. Other agonists (specific for the endothelium) were not used in the present study because the size of the monkeys and their coronary arteries precluded extensive experimental protocols. Regardless of the mechanisms, results of the current experiment provide new information about the determinants of coronary artery reactivity in premenopausal females.

Conclusions

The objective of the present study was to define important determinants of coronary artery reactivity among premenopausal female monkeys with coronary artery atherosclerosis. Of the modulators and risk factors examined, social status (and resulting psychosocial stress) and plasma estradiol concentrations measured on the same day as coronary artery reactivity were the most important modulators of acetylcholine-mediated dilation. Such information may be useful in identifying risk factors and modulators of CHD in women.

Acknowledgments

This research was supported in part by grants RO1-HL39789 (Dr Shively) and PO1-HL45666, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md. We thank Jamie Fox for his expert technical assistance and Karen Potvin Klein for her editorial contributions.

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Determinants of coronary artery reactivity in premenopausal female cynomolgus monkeys with diet-induced atherosclerosis.
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Circulation. 1994;90:983-987
doi: 10.1161/01.CIR.90.2.983

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