Gender Gap in Aortic Cholesterol Accumulation in Cholesterol-Clamped Rabbits
Role of the Endothelium and Mononuclear-Endothelial Cell Interaction

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Background—The purpose of the present study was to investigate plasma lipid–independent mechanisms for the sex difference in the development of atherosclerosis.

Methods and Results—In the first experiment, 20 male and 20 female rabbits were balloon-injured in the middle thoracic aorta and maintained at the same plasma cholesterol level of ≈25 mmol/L by use of individualized cholesterol feeding for 13 weeks. In the undamaged aorta, female rabbits had accumulated less than half the amount of cholesterol found in male rabbits (P<0.05). In the balloon-injured aorta, cholesterol accumulation was 3- to 4-fold higher than in the undamaged aorta, with no difference between groups. When cholesterol accumulation data for the balloon-injured aorta were separately assessed for blue (deendothelialized) and white (reendothelialized) tissue, blue tissue surprisingly revealed a reverse gender gap, ie, a significantly higher accumulation of cholesterol in females than in males (P<0.05). White tissue, which constituted the majority of the balloon-injured area, showed no difference in aortic cholesterol accumulation between groups. In the second experiment, 6 male and 6 female rabbits were fed standard rabbit pellets and 6 male and 6 female rabbits were fed a 0.5% cholesterol-enriched chow for 2 weeks. Mononuclear cell binding was 5-fold higher in aortic segments from hypercholesterolemic than from normocholesterolemic rabbits (P<0.001). In hypercholesterolemic rabbits, cell binding was significantly lower in female than in male rabbits (P<0.05) and showed higher values in atherosclerosis-prone regions. These differences were not found in normocholesterolemic animals.

Conclusions—The present results suggest that female atheroprotection is independent of sex differences in plasma cholesterol but vitally dependent on the state of the arterial endothelium and involves mononuclear-endothelial cell adhesion as an early step. (Circulation. 1998;98:2731-2737.)

Key Words: aorta ▪ atherosclerosis ▪ balloon ▪ endothelium ▪ leukocytes

Coronary atherosclerosis is the leading cause of death in the western world.1 One of the most important risk factors for this disease is male sex, with a male-to-female ratio of death of coronary disease close to 4 before the age of 50.2 This sex difference is not eliminated by mathematical adjustments for sex differences in the prevalence of classic risk factors such as hyperlipidemia, hypertension, diabetes, smoking, and obesity.3 An inborn difference in arterial wall structure has been suggested, but results are controversial.4,5

The male-to-female ratio of death due to coronary disease declines gradually from the age of female menopause.5 Furthermore, a variety of epidemiological studies suggest that the risk of coronary disease is increased in ovariectomized and postmenopausal women compared with age-matched menstruating women6,7 and conversely is decreased in women taking estrogen replacement therapy compared with age-matched controls.8,9 Thus, a likely explanation for the sex difference in coronary disease prevalence is the presence of higher circulating blood levels of estrogen in women compared with men. On the other hand, evidence exists that men treated with estrogen, either as primary or secondary prophylaxis, experience an excess number of cardiovascular events compared with their nontreated counterparts.10,11 Furthermore, the endogenous levels of estrogen in men are only poorly correlated with coronary disease.2

In humans, the sex difference in atherosclerosis extent is present in the coronary arteries but not in the aorta.12 It has previously been shown that monkeys and rabbits fed a cholesterol-enriched diet also show a higher extent of atherosclerosis in males than in females.13,14 In the rabbit study, this difference was demonstrated in aortic tissue, which suggests that the rabbit aorta is a better model for coronary than for aortic atherosclerosis in humans. In that study, the male-female difference in atherosclerosis extent was present despite similar plasma cholesterol levels, which suggests a direct action of female sex at the level of the arterial wall. The purpose of the present study was to investigate whether a direct atheroprotective effect of female sex (1) depends on the state of the arterial endothelium (atherosclerosis experiment) and (2) is preceded by differences in endothelial adhesiveness.
for mononuclear cells (mononuclear cell adhesion experiment).

**Methods**

All animals were sexually mature rabbits of the Danish Country Strain obtained from Statens Serum Institute, Copenhagen. The rabbits were housed in individual stainless steel cages at a room temperature of 20°C and with a 12-hour light cycle. The animals had free access to drinking water. All experimental procedures were performed in accordance with Danish regulations for experiments with animals.

**Atherosclerosis Experiment**

**Experimental Design**

Twenty male and 20 female rabbits were fed a cholesterol-enriched diet of chow (Altromin 2113) for 13 weeks (80 g/d). Cholesterol (Sigma Chemical Co) was added to the chow in 4 different concentrations: 0%, 0.25%, 0.5%, and 1% wt/wt.17–20 Guided by weekly plasma cholesterol determinations (CHOD-PAP, Boehringer Mannheim), we maintained all animals at a plasma cholesterol level of ~25 mmol/L using the 4 different chow preparations.17–20

After 1 week of cholesterol feeding, the rabbits were anesthetized with intravenous pentobarbital (50 mg/kg body weight) and underwent balloon catheter injury in the middle thoracic aorta as described previously.17–20 The postoperative mortality rate was 0%.

After 12 weeks of cholesterol feeding, blood samples were drawn for determination of the distribution of cholesterol among lipoprotein fractions.17,18 One week later, the rabbits were injected with 5 mL of Evans blue dye (5 mg/mL), which was allowed to circulate for 5 minutes before the rabbits were killed with an overdose of intravenous pentobarbital (50 to 100 mg/kg body weight).17–20 The aorta was then removed from the level of the aortic valves to the level of the diaphragm.

**Preparation of Aortic Tissue**

The aorta was cleaned of adherent adventitial tissue, opened longitudinally, and divided into 3 parts: 1 part extended from the aortic valves to the second intercostal arteries (arch plus upper thoracic; undamaged), 1 from the second to the fifth intercostal arteries (middle thoracic; balloon injured), and 1 from the fifth intercostal arteries to the celiac artery (lower thoracic; undamaged). The balloon-injured middle thoracic aorta was further separated into white tissue, consisting of reendothelialized areas, and blue tissue, consisting of still deendothelialized areas. Each aortic part was fixed with pins on a cork board, and the inner layer containing the intima and part of the media was stripped from the outer media. The outer layer was discarded, and the inner layer was weighed and stored within 1 hour at ~20°C until analyzed.

**Evaluation of Atherosclerosis Extent**

Aortic cholesterol was determined as described previously.18–20 In brief, the aortic tissue was minced and extracted with ~20 vol of chloroform/methanol for 24 hours. The extract was washed by the Folch procedure, and the cholesterol content was determined enzymatically after evaporation and redissolution in isopropanol. The validity of this procedure has been tested previously.21

**Mononuclear Cell Adhesion Experiment**

**Experimental Design**

Six male and 6 female rabbits were fed standard rabbit pellets, and 6 male and 6 female rabbits were fed a 0.5% cholesterol-enriched chow. After 2 weeks, blood samples were drawn for determination of plasma cholesterol levels, after which the rabbits were killed by an overdose of intravenous pentobarbital. The aorta was removed, placed in cold PBS, and carefully freed of adventitia.

**Isolation and Fluorescence Labeling of Mononuclear Cells**

Whole human blood was retrieved by venous puncture in sodium citrate (10:1 vol/vol) and heparin (10 U/mL). The blood was carefully layered onto a cushion of Lymphoprep (Nycomed Pharma A/S) and centrifuged at 782g for 30 minutes at room temperature. The buffy coat was removed and washed twice with RPMI 1640 medium (ATTC). The resulting cell pellet was allowed to incubate in RPMI 1640 medium containing tetramethylrhodamine isothiocyanate 3 μg/mL (TRITC; CalBiochem) for 15 minutes at room temperature. To separate labeled cells from the remaining dye, the cell suspension was carefully underlaid with a layer of fetal calf serum and centrifuged at 425g for 10 minutes at room temperature. The cells were finally washed in RPMI 1640 medium and resuspended in HBSS (Life Technologies) containing 2 mmol/L Ca++, 2 mmol/L Mg++, and 20 mmol/L HEPES for binding studies.

**Binding Assay**

The binding assay was performed as described previously by Tsao et al.22 Segments (~15 mm) were excised from the level of the aortic arch (immediately below the carotid arteries), the thoracic aorta (sewn to third intercostal arteries), and the abdominal aorta (celiac to superior mesenteric artery). The segments were opened longitudinally and, with the endothelial side up, were affixed with needles to 35-mm culture dishes containing 2 mL of HBSS. The dishes were then placed on a rocking platform at room temperature.

After 10 minutes, the HBSS medium was replaced by binding medium containing mononuclear cells at a concentration of 5×10⁶ cells/mL. The aortic segments were incubated with the mononuclear cells for 30 minutes, after which the medium was aspirated and the segments washed twice with fresh binding medium to remove nonadherent cells. After an additional 5 minutes on the rocking platform, the aortic segments were removed and placed on a glass slide with the endothelial side up. Adherent cells were counted under fluorescence microscopy from 9 predetermined, equally distributed sites on each segment. The identification of the segments was revealed after all segments were counted.

**Statistics**

In the atherosclerosis experiment, comparisons between groups were made by use of the Wilcoxon U test for unpaired samples. In the mononuclear cell adhesion experiment, we evaluated differences between groups by a 2-way ANOVA with post hoc analysis using the Newman-Keuls test. In Figure 5, data were analyzed by the Kruskal-Wallis test followed by a Dunn’s multiple comparison test. P<0.05 was considered statistically significant. All analyses were performed with the GraphPad Prism software program.

**Results**

**Atherosclerosis Experiment**

**Characteristics of Female and Male Rabbits**

Initial body weights were similar in the female and male rabbit groups. Both groups thrived and gained weight during the study period. The female group, however, had a significantly greater weight gain than the male group (Table). The initial plasma cholesterol concentration was significantly higher in the female than in the male rabbits. This difference was eliminated by cholesterol feeding, which elevated plasma cholesterol to a similar level of ~25 mmol/L in 31 rabbits. The remaining 9 animals (6 males and 3 females) had lower plasma cholesterol concentrations throughout the study either because of anorexia or because of a pronounced resistance to the development of diet-induced hypercholesterolemia. These rabbits were excluded from the study. The amount of dietary cholesterol, which was used to adjust each rabbit’s plasma cholesterol to 25 mmol/L, was not different between groups. The mean exposure of plasma cholesterol to the aortic wall, which can be calculated as the area under the time versus plasma cholesterol curve, divided by the duration of the experiment, was similar in the female and male rabbits at the end of the experiment (Table). The female rabbits
had a significantly higher level of VLDL and a significantly lower level of LDL than the male rabbits, but none of these parameters were, within the observed values, statistically significantly related to aortic cholesterol accumulation.

**Aortic Atherosclerosis**

In undamaged aortas, ie, the aortic arch plus upper thoracic aorta and the lower thoracic aorta, female rabbits had only one half and one third, respectively, the accumulation of cholesterol found in male rabbits (P<0.05) (Figure 1). In the balloon-injured middle thoracic aorta, cholesterol accumulation was 3- to 4-fold higher than in the undamaged aorta and was not different between the 2 groups. When the cholesterol accumulation data for the balloon-injured area were separately assessed for blue (deendothelialized) and white (reendothelialized) tissue, whereas only a minor part consisted of deendothelialized blue tissue (0.20±0.03 and 0.16±0.03 blue tissue mg wet wt /total tissue mg wet wt for females and males, respectively).

**Mononuclear Cell Adhesion Experiment**

At the end of the 2-week experiment, female rabbits had a higher plasma cholesterol level than male rabbits in the normocholesterolemic group (0.8±0.2 versus 0.4±0.1 mmol/L; P<0.05), whereas there was no significant difference between female and male rabbits in the hypercholesterolemic group (12.7±1.3 versus 10.0±1.1 mmol/L).

Figure 3 shows photomicrographs of aortic segments from normocholesterolemic (A), hypercholesterolemic male (B), and hypercholesterolemic female (C) rabbits. Aortic segments from normocholesterolemic rabbits demonstrated only minimal binding of mononuclear cells to the endothelial cells. There was no difference in cell binding either between female and male rabbits or among the different aortic segments within each group (Figure 4). Aortic segments from hypercholesterolemic rabbits, however, showed a 5-fold increase in cell binding compared with normocholesterolemic rabbits (P<0.001) (Figure 4). This increase was significantly attenuated in the female compared with the male group as determined by a 2-way ANOVA (P<0.05). There was also a regional variation in the magnitude of cell binding: the aortic arch bound a higher amount of mononuclear cells than the thoracic aorta (P<0.05), which bound a higher amount of mononuclear cells than the abdominal aorta. The latter difference, however, was only marginally significant (P<0.10).

**Discussion**

**Atherosclerosis Experiment**

It was possible to maintain plasma cholesterol at ≈25 mmol/L for 13 weeks in 31 of 40 animals. In these

![Figure 2. Extent of aortic cholesterol accumulation for balloon-injured area, shown separately for blue (deendothelialized) and white (reendothelialized) tissue. P values are for comparisons between male (solid bar, n=14) and female (hatched bar, n=17) rabbits by Mann-Whitney U test. Data are mean±SEM. *P<0.05.](image)

![Figure 1. Extent of aortic cholesterol accumulation for undamaged and balloon-injured areas. P values are for comparisons between male (solid bar, n=14) and female (hatched bar, n=17) rabbits by Mann-Whitney U test. Data are mean±SEM. *P<0.05.](image)
animals, the amount of dietary cholesterol necessary to maintain the elevated plasma cholesterol was without significant difference between the 2 groups, which suggests a neutral effect of female sex on total plasma cholesterol in cholesterol-fed rabbits. Aortic accumulation of cholesterol in the females was less than half that in the males. These results are in accordance with previous findings\textsuperscript{16} that showed an identical elevation of plasma cholesterol in female and male rabbits fed a fixed (2\%) cholesterol-enriched diet for several weeks and significantly less aortic atherosclerosis in female

![Figure 3](image-url)

**Figure 3.** Photomicrographs of aortic segments demonstrating differences in mononuclear cell adhesion between normocholesterolemic (A), hypercholesterolemic male (B), and hypercholesterolemic female (C) animals.

![Figure 4](image-url)

**Figure 4.** Mononuclear-endothelial cell binding, as determined by an ex vivo assay, in normocholesterolemic (top) and hypercholesterolemic (bottom) rabbits. Comparisons between male (solid bar, n=6×2) and female (hatched bar, n=6×2) rabbits and between the different aortic segments were made by use of 2-way ANOVA. Data are mean±SEM.

*P*<0.05; (*)P<0.10.
than in male rabbits after 10 weeks. After 15 weeks, however, this sex difference was no longer present. These findings suggest that the direct, ie, plasma lipid–independent, athero-protective effect of female sex involves a retardation rather than an inhibition of the development of atherosclerosis.

To further explore this direct atheroprotection by female sex, we subjected all rabbits to balloon catheter injury in the middle thoracic aorta. We 19 have previously shown that this procedure results in complete endothelial denudation, whereas the deeper arterial layers, ie, the internal elastic lamina and the smooth muscle of the media, are left intact. At necropsy 12 weeks after surgery, the balloon-injured area consists partly of areas denuded of endothelium and partly of areas covered with regenerated endothelial cells that are irregularly shaped, lack alignment in the direction of the blood flow, and exhibit endothelial dysfunction.23 The present findings, which show lower cholesterol accumulation in female than in male rabbits in the intact aorta, similar cholesterol accumulation in the 2 groups in the aorta with regenerated endothelium, and higher accumulation in the female than in the male group in the aorta denuded of endothelium, suggest that the direct atheroprotective effect of female sex is dependent on the state of the arterial endothe- lium. If such a dependence relies on upregulation of an endothelial antiatherogenic factor, the findings furthermore suggest that this occurs along with upregulation of other vascular, potentially atherogenic factors that eliminate or overrule protection in aortas with dysfunctioning or absent endothelium. To the best of our knowledge, effects of gender on cholesterol accumulation after balloon catheter injury have not previously been investigated in a hypercholesterolemic animal model.

Mononuclear Cell Adhesion Experiment

The finding of 5-fold higher binding of mononuclear cells to the endothelium of vascular segments from hypercholesterolemic rabbits compared with that of normocholesterolemic rabbits is consistent with previous observations.22 Adherence of mononuclear cells to the vascular endothelium is one of the earliest detectable abnormalities in the arteries of hypercholesterolemic animals.24 The adherent cells infiltrate the sub-intima and transform into lipid-engorging macrophages, result- ing in the development of foam cells and fatty streaks, the first gross pathological lesion of atherosclerosis. The exact mechanism that initiates this mononuclear-endothelial cell interaction is not known, but expression of endothelial adhesion molecules and chemotactic proteins induced by hypercholesterolemia may be involved.25,26

The long-term cholesterol-fed rabbit exhibits a characteristic regional pattern in the development of atherosclerosis: the lipid lesions are most pronounced in the aortic arch and involve smaller areas in the more distal parts of the aorta.17–20,27,28 The present findings showing a similar regional pattern in mononuclear-endothelial cell binding in hypercholesterolemic rabbits are supportive of a relation between an early (weeks) increase in endothelial adhesiveness and later (months) development of lipid lesions in these animals. Thus, the significantly lower mononuclear-endothelial cell binding found in the aortas of female compared with male hypercho- lesterolemic rabbits may play a role in the mediation of the endothelium-dependent sex difference in aortic cholesterol accumulation described in the atherosclerosis experiment. The mechanism by which female sex is able to affect endothelial adhesiveness is not known. It has previously been shown that basal release of endothelial nitric oxide (NO) is significantly higher in aortic rings from female than from male normocholesterolemic rabbits29 and that endothelial constitutive NO synthase (eNOS), the enzyme that catalyzes NO formation, is upregulated in pregnant and estrogen-treated guinea-pigs.30 In a recent study, mononuclear-endothelial cell interaction was increased by long-term adminis-tration of an NO synthase antagonist and conversely inhibited by long-term administration of the NO precursor L- arginine in cholesterol-fed rabbits, which suggests that NO is able to regulate endothelial adhesiveness for mononuclear cells.22 An endothelial factor that is regulated by female sex and that affects endothelial adhesiveness could thus be identical to NO. This relationship, however, was not addressed in the present study and warrants further investigation.

Importance of Estrogen

We 20 have previously conducted a rabbit study of the same duration and with the same plasma cholesterol levels as in the atherosclerosis experiment performed in the present study; the previous study consisted of intact males, intact males treated with estrogen, ovariectomized females, and ovariectomized females treated with estrogen. Because the male rabbits of that experiment accumulated nearly the same amount of cholesterol in the thoracic aorta as the male rabbits of the present experiment (26 and 21 nmol/mg wet weight, respectively), we compared the results of the 2 experiments and found a consistent pattern in the accumulation of cholesterol. As shown in Figure 5 (top), the gender gap in atherosclerosis development is abolished when males are treated with estrogen. When females are ovariectomized, which eliminates their endogenous estrogen production, their protection is lost, and when they are given estrogen replacement, their protection is regained. Interestingly, the difference in atherosclerosis extent between intact and ovariectomized females may be more prominent when females are kept in the same room as males, as in the present study, than when intact and ovariectomized females are kept apart.27,33,32 Probably, the presence of males increases the endogenous production of estrogen in females. The reason ovariectomized females tend to develop even more atherosclerosis than male rabbits (P<0.10) is not known, but the same trend is seen in monkeys fed an atherogenic diet.15 One possibility is that males, with their low endogenous estrogen production, are protected relative to ovariectomized females that are completely devoid of estrogen. As can be seen in Figure 5 (bottom), denudation of the aortic endothelium by balloon catheter injury elimi-nates all differences in aortic cholesterol accumulation be-tween the groups. Taken together, these results suggest that the direct atheroprotection conferred by female sex, demonstrated above, is explained exclusively by the presence of higher estrogen concentrations in females than in males and that this effect of estrogen depends on an intact endothelium.
Results from previous experimental animal studies investigating the effect of estrogen on balloon catheter–induced atherosclerosis are controversial. In normocholesterolemic rats, mice, and rabbits, estrogen protects against neointimal hyperplasia after balloon catheter injury. It has been suggested that this effect is mediated by a facilitation of the reendothelialization and thus the functional endothelial recovery of the damaged artery. In hypercholesterolemic rabbits and monkeys, estrogen has no effect neither on neointimal hyperplasia or on cholesterol accumulation after balloon catheter injury. One explanation for this discrepancy between effects of estrogen on balloon catheter–induced atherosclerosis in normocholesterolemic and hypercholesterolemic animals is that hypercholesterolemia impairs reendothelialization and thus counteracts or abolishes the beneficial effect of estrogen.

In the present study, we observed a surprisingly higher cholesterol accumulation in female than in male rabbits in the deendothelialized aorta. A paradoxical atherogenic effect of estrogen has previously been observed in balloon-injured aorta of rabbits treated with estrogen plus N\textsubscript{G}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO production. Thus, absence of endothelial NO, either by enzyme inhibition or by endothelial denudation, may play a role in this previously unnoticed effect of estrogen/female sex on vascular tissue.

**Conclusions**

This study confirms a previous finding that female rabbits develop less-extensive diet-induced atherosclerosis than males. More importantly, it suggests that this relative protection of female rabbits is independent of sex differences in plasma cholesterol and lipoproteins but vitally dependent on the state of the arterial endothelium, because it is present in aortas with intact endothelium, absent in aortas with regenerated endothelium, and even reversed in aortas denuded of endothelium. An endothelium-dependent mechanism for the gender gap in the development of atherosclerosis may involve mononuclear-endothelial cell binding, which is significantly greater in aortic segments from female than from male rabbits even after just 2 weeks of cholesterol feeding and which shows a regional variation similar to that seen for atherosclerosis.

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References

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