

Significant Reduction of the Antiatherogenic Effect of Estrogen by Long-term Inhibition of Nitric Oxide Synthesis in Cholesterol-clamped Rabbits

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Abstract

The purpose of this study was to investigate whether endothelium-derived nitric oxide (NO) is involved in the plasma lipid-independent antiatherogenic effect of estrogen and levormeloxifene, a partial estrogen receptor agonist. 85 rabbits were ovariectomized and balloon-injured in the middle thoracic aorta. The rabbits were fed a cholesterol-enriched diet supplemented with 17 β -estradiol, levormeloxifene, or placebo, either alone, or together with 160 μ g/ml N^G-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor, in their drinking water for 12 wk. Plasma cholesterol was maintained at 25–30 mmol/liter by individualized cholesterol feeding.

In the undamaged aorta, the extent of atherosclerosis in the estrogen group was only one-third that in the placebo group. Simultaneous administration of L-NAME, however, significantly reduced the antiatherogenic effect of estrogen ($P < 0.01$). There was no significant difference between the placebo group given L-NAME and the group treated with placebo alone. At the previously endothelium-denuded site, estrogen had no effect on atherosclerosis development, whereas L-NAME combined with estrogen significantly increased atherogenesis ($P < 0.05$). The effects of levormeloxifene were almost similar to those of estrogen. Active vascular concentrations of L-NAME were demonstrated in an additional study, in which maximal aortic/coronary endothelium-dependent relaxation was significantly inhibited in rabbits given L-NAME.

Thus, in this study a considerable part of the plasma lipid-independent antiatherogenic effect of estrogen was mediated through its effect on endothelial NO in cholesterol-fed rabbits. The results for levormeloxifene suggest a common mechanism of action for estrogen and partial estrogen receptor agonists on atherogenesis. (*J. Clin. Invest.* 1997. 100:821–828.) Key words: 17 β -estradiol • levormeloxifene • atherosclerosis • nitric oxide • endothelium

Introduction

Cardiovascular disease is less prevalent in premenopausal women and women receiving estrogen replacement therapy than in postmenopausal women or men (1–3). Although data from studies with randomized allocation for treatment are still lacking, the notion of a protective effect of estrogen against cardiovascular disease is further supported by a number of experimental animal studies (4–8). One potential mechanism for the cardioprotective effect of estrogen is through its beneficial effects on plasma lipoproteins (9). It is becoming increasingly clear, however, that these changes account for the cardioprotective effect only to a limited extent (10).

Direct actions of estrogen on the arterial wall seem more important. First, long-term administration of estrogen inhibits arterial cholesterol deposition and intimal thickening independent of changes in plasma lipoproteins (4–8) in ovariectomized cynomolgus monkeys and rabbits fed an atherogenic diet. Second, short-term (20 min) and long-term (2 yr) administration of estrogen modulates responses to acetylcholine in atherosclerotic coronary arteries of ovariectomized cynomolgus monkeys (11, 12). Similar effects have been observed in women after short-term administration of estrogen (13–15).

Whereas the direct antiatherogenic effect of estrogen on the arterial wall is only poorly understood, the hemodynamic effects are believed to be mediated, at least in part, through the ability of estrogen to increase endothelial nitric oxide (NO)¹ synthesis (16–18). NO plays a key role in the regulation of blood flow and pressure (19), but may also suppress a number of processes involved in atherosclerosis, such as platelet adhesion and aggregation, vascular smooth muscle cell proliferation, and monocyte–endothelial cell interactions (20). It has therefore been hypothesized that the ability of estrogen to increase endothelial NO synthesis is also involved in the antiatherogenic effect of estrogen (18, 21), but more direct evidence is lacking. We have shown previously that antiatherogenicity of estrogen is abolished by balloon catheter injury in cholesterol-clamped rabbits, suggesting that an intact endothelium is of vital importance for estrogen actions on the arterial wall (22, 23). Similar findings were obtained with levormeloxifene, a partial estrogen receptor agonist (Holm et al., manuscript submitted for publication).² In this study we investigate whether the antiatherogenic effects of estrogen and levormeloxifene are attenuated by simultaneous treatment with N^G-nitro-L-arginine methyl ester (L-NAME), an NO synthase (NOS) inhibitor, in undamaged and balloon-injured rabbit aorta.

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1. Abbreviations used in this paper: L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase.

2. Holm, P., M. Shalmi, N. Korsgaard, B. Gulddammer, S.O. Skouby, and S. Stender, manuscript submitted for publication.

Methods

Animals

Mature female New Zealand White rabbits (Interfauna, Huntingdon, England) were housed individually at a room temperature of $18 \pm 2^\circ\text{C}$ with a 12-h light cycle and free access to drinking water. All experimental procedures were performed in accordance with the Danish regulations for experiments with animals.

Experimental design

85 rabbits were given a cholesterol-enriched chow for 13 wk (80 g/d), and were maintained at the same plasma cholesterol level of 25–30 mmol/liter by use of an individualized cholesterol feeding protocol (22, 23).

After 1 wk of cholesterol feeding, the rabbits were anesthetized with intravenous pentobarbital (50 mg/kg body wt). Ovariectomy and balloon catheter injury were performed as described previously (5, 22, 23), except that the endothelial denudation was made in the middle thoracic aorta (second to fifth intercostal arteries). The length from the site of insertion of the catheter in the right iliac artery to the second intercostal arteries was determined by x ray in two rabbits of similar size as those used in the main study.

Immediately after surgery, the rabbits were divided into three groups with similar baseline (i.e., preexperimental) values of plasma cholesterol and body weight to receive daily oral treatment with 17β -estradiol (Sigma Chemical Co., St. Louis, MO) (4 mg/d), levormeloxifene (10 mg/d), or placebo (vehicle) (5). By use of a similar stratification protocol as above, the animals in each of the three groups were divided into two sets: those drinking untreated water, and those drinking water containing 160 $\mu\text{g/ml}$ L-NAME (Sigma Chemical Co.). L-NAME was administered throughout the study period.

After 8 wk of L-NAME administration, intraarterial blood pressure was measured in all rabbits,² and blood samples were drawn for determination of the distribution of cholesterol between lipoprotein fractions (22, 23).

At the end of the experiment, the rabbits were injected intravenously with Evans Blue dye, killed with an overdose of intravenous pentobarbital, and their systemic circulation was perfused with saline (22, 23). Subsequently, the aortas were dissected from the aortic valves to the level of the diaphragm.

Tissue preparation

Aortic cholesterol. The aorta was opened longitudinally and divided into the balloon-injured middle thoracic aorta and three undamaged sites, i.e., the aortic arch (aortic valves to ductus arteriosus), and the upper (ductus arteriosus to second intercostal arteries) and lower (fifth intercostal arteries to celiac arteries) thoracic aorta. The balloon-injured site was easily identified, appearing as blue areas of still deendothelialized tissue and elevated white pads of reendothelialized tissue. Each aortic site was fixed with pins on a corkboard, and the inner layer containing the intima and part of the media was stripped from the outer media, weighed, and stored within 1 h at -20°C until analyzed.

Histomorphometry. Before opening the aorta, rings were taken from the central part of the balloon-injured middle thoracic aorta and the undamaged aortic arch. The rings were fixed in neutral buffered formalin, embedded in paraffin, sectioned (one section/aortic ring), and stained with hematoxylin and eosin.

Evaluation of atherosclerosis extent

Aortic cholesterol was extracted with chloroform/methanol (1:1; vol/vol), and was determined enzymatically after evaporation and dissolution in isopropyl alcohol (6). Intimal thickness was evaluated by projecting images of the entire hematoxylin and eosin-stained aortic rings onto the screen of a computer through a video-monitored microscope (Olympus Corp., Lake Success, NY). The number of grid points overlying the intima and media was counted by an independent and blinded observer, and the ratio was calculated.

Vascular reactivity studies

Vascular reactivity studies were not performed in the main study because Evans Blue dye acts as an inhibitor of soluble guanylyl cyclase, thus preventing the production of cGMP and relaxation (24). Therefore, an additional 18 rabbits were ovariectomized. After 1 wk of recovery, the rabbits were divided into three groups of six rabbits each: one group drinking untreated water, and two groups drinking water containing either 160 or 320 $\mu\text{g/ml}$ L-NAME. L-NAME was administered for 1 wk, after which the rabbits were killed with an overdose of intravenous pentobarbital.

Aorta. A ring from the upper thoracic aorta, free of periaortic tissue, was within 5 min suspended in individual organ baths for the

Table I. Body Weights, Cholesterol Values, and Hemodynamic Variables of the Six Rabbit Groups

	Estrogen		Levormeloxifene		Placebo		ANOVA
	–L-NAME	+L-NAME	–L-NAME	+L-NAME	–L-NAME	+L-NAME	
No. of animals	14	13	13	13	18	14	
Body wt (kg)							
Baseline	3.5 \pm 0.1	3.4 \pm 0.1	3.5 \pm 0.1	3.3 \pm 0.1	3.5 \pm 0.1	3.4 \pm 0.1	NS
Final	3.8 \pm 0.1*	3.5 \pm 0.1	3.4 \pm 0.1	3.3 \pm 0.1	3.5 \pm 0.1	3.5 \pm 0.1	$P < 0.01$
Total plasma chol (mmol/liter)							
Baseline	0.9 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1	1.0 \pm 0.1	0.9 \pm 0.1	0.9 \pm 0.1	NS
AUC/91 d	25.7 \pm 0.4	26.0 \pm 0.6	26.9 \pm 0.8	28.5 \pm 1.0	26.2 \pm 0.6	27.4 \pm 0.7	NS
Dietary chol total intake (g)	39 \pm 3 [‡]	36 \pm 3	46 \pm 4 [§]	38 \pm 4	28 \pm 2	29 \pm 3	$P < 0.0001$
Lipoproteins at wk 8 (% of total)							
VLDL	54 \pm 2	54 \pm 2	61 \pm 2	54 \pm 2	53 \pm 2	54 \pm 2	NS
IDL	32 \pm 1	34 \pm 1	28 \pm 2	32 \pm 2	30 \pm 1	30 \pm 1	$P < 0.05$
LDL	11 \pm 1*	10 \pm 1	10 \pm 1 [‡]	12 \pm 1	15 \pm 1	15 \pm 2	$P < 0.02$
HDL	3 \pm 0	3 \pm 0	2 \pm 0	2 \pm 0	3 \pm 0	2 \pm 0	NS
SBP at wk 8 (mmHg)	86 \pm 2*	90 \pm 2	84 \pm 2*	87 \pm 2	93 \pm 2	87 \pm 2	$P < 0.05$
DBP at wk 8 (mmHg)	57 \pm 2	59 \pm 2	55 \pm 2	60 \pm 2	61 \pm 1	59 \pm 2	NS

All values are given as mean \pm SEM. Chol, cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; AUC, area under curve, determined by the trapezium rule (27). * $P < 0.05$, [‡] $P < 0.005$, [§] $P < 0.0005$ vs. placebo group. ^{||} $P < 0.05$ vs. placebo +L-NAME group. No significant differences were found within groups treated or nontreated with L-NAME.

measurement of isometric tension as described previously (25). After precontraction with phenylephrine (4×10^{-7} M), cumulative dose-response curves for acetylcholine and sodium nitroprusside (10^{-8} – 10^{-5}) were performed.

Coronary artery. The heart was removed and immediately immersed in ice-cold Krebs' solution. A segment of 4 mm from the septal artery, free of connective tissue, was within 5 min mounted in a myograph for the measurement of isometric tension as described previously (26). The vessels were precontracted with potassium (30 mM), and their ability to relax to acetylcholine and sodium nitroprusside was determined as above, except that the measurements for acetylcholine were performed in the presence of indomethacin (10^{-6} M), an inhibitor of prostanoïd synthesis.

Statistics

ANOVA was used to test for statistically significant differences between the groups with regard to the values in Table I. If ANOVA indicated significant differences, Student's *t* test was used to compare treatments.

The aortic cholesterol data (see Fig. 1) were analyzed for all tissues combined by a mixed model. This is an analysis similar to the ANOVA, but with two variance components: a between rabbit variation, and a within rabbit variation (error). Differences between treatments are compared to the variation between rabbits, whereas tissue effects are compared to the smaller error variation. Insignificant interactions and effects were excluded from the model. The final model included an effect of tissue, an interaction between hormone treatment and NO treatment, and an interaction between hormone treatment and tissue damage. As the effects were expected to be multiplicative, the analysis was done on a logarithmic scale. Final results are reported as multiplicative effects (factors) on the original scale. Confidence intervals (95%) were made for the effect on the original scale. The software used was SAS (proc mixed; SAS Institute, Inc., Cary, NC). The intimal/medial cross-sectional areas (see Fig. 2) were evaluated by ANOVA, separately for the undamaged aortic arch and the balloon-injured middle thoracic aorta.

The vascular reactivity data (see Figs. 3 and 4) were compared by use of Student's *t* test. All values are given as mean \pm SEM.

Results

Characteristics of the six rabbit groups

After a postoperative recovery period of 3–4 d, all rabbits thrived well and showed no side effects of treatment or cholesterol feeding (Table I). Because of the protocol for cholesterol clamping, plasma cholesterol levels were greatly and similarly elevated in all groups (Table I). The similar plasma cholesterol levels, however, were obtained at the expense of the amount of total dietary cholesterol, which was significantly higher for the estrogen/levormeloxifene group than for the placebo group, and was significantly higher for the estrogen/levormeloxifene+L-NAME group than for the placebo+L-NAME group. The distribution of cholesterol between lipoprotein fractions showed only minor changes between groups. Likewise, systolic and diastolic blood pressure was almost similar in all groups. Neither of the values in Table I showed significant changes within estrogen, levormeloxifene, and placebo groups, demonstrating that the values were unaffected by L-NAME treatment.

Extent of atherosclerosis

Aortic cholesterol content. In rabbits not treated with L-NAME, the estrogen and levormeloxifene group had accumulated significantly less cholesterol than the placebo group in all undamaged sites of the aorta; overall, the cholesterol accumulation

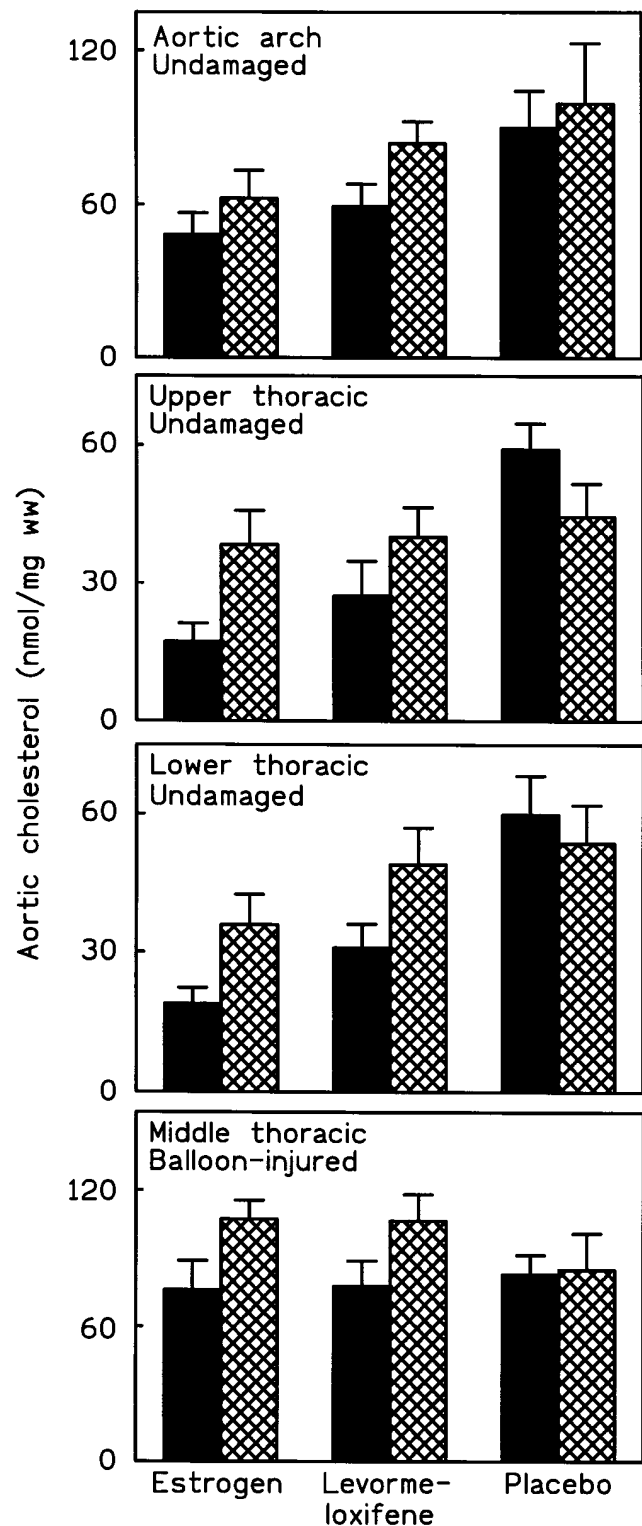


Figure 1. Aortic cholesterol accumulation of the six groups. Estrogen had a significant antiatherogenic effect in the undamaged aorta; this effect was abolished by balloon catheter injury, and was significantly attenuated by simultaneous administration of L-NAME. Similar findings, although not as pronounced, were obtained for levormeloxifene. All values are mean \pm SEM. For statistics, see Table II. Black bars, -L-NAME; hatched bars, +L-NAME.

Table II. Estimate of Combined Effect of Hormone Treatment and L-NAME Treatment in Mixed Model. Multiplicative Effects, Relative to Placebo Without L-NAME Treatment

	Estrogen		Levormeloxifene		Placebo	
	-L-NAME	+L-NAME	-L-NAME	+L-NAME	-L-NAME	+L-NAME
Undamaged tissue	0.371 [‡] (0.260–0.529)	0.635 (0.442–0.911)	0.539* (0.375–0.774)	0.854 (0.594–1.227)	1 (0.632–1.264)	0.894 (0.632–1.264)
Balloon-injured tissue	0.801 (0.534–1.202)	1.370 [§] (0.907–2.069)	0.845 (0.559–1.277)	1.338 [§] (0.885–2.024)	1 (0.632–1.264)	0.894 (0.632–1.264)

Confidence intervals are given in parentheses. * $P < 0.005$, [‡] $P < 0.0005$ vs. placebo group; [§] $P < 0.05$ vs. placebo +L-NAME group; ^{||} $P < 0.05$ vs. corresponding group treated with L-NAME.

was reduced to one-third in the estrogen group, and to one-half in the levormeloxifene group (Fig. 1 and Table II). The antiatherogenic effect of estrogen, however, was reduced significantly by the addition of L-NAME (estrogen group vs. estrogen+L-NAME group; $P < 0.01$ by a mixed model analysis for undamaged tissue). A similar trend was seen for levormeloxifene. This resulted in no significant reduction of atherosclerosis by treatment with estrogen/levormeloxifene in combination with L-NAME as compared with the placebo group treated with L-NAME in the undamaged aorta ($P = 0.08$) (Fig. 1 and Table II). The addition of L-NAME to placebo treatment did not significantly change the aortic accumulation of cholesterol.

In the balloon-injured site, estrogen and levormeloxifene had no significant effect on aortic cholesterol accumulation compared with the placebo group (Fig. 1 and Table II). Treatment with estrogen/levormeloxifene in combination with L-NAME significantly increased the aortic accumulation of cholesterol compared with the placebo group treated with L-NAME ($P < 0.05$). Again, the addition of L-NAME to placebo treatment did not significantly affect aortic cholesterol accumulation.

Histomorphometry. A blinded evaluation of intimal/medial cross-sectional areas revealed the same picture as above (Fig. 2), with a pronounced antiatherogenic effect of estrogen/levormeloxifene treatment compared to placebo treatment ($P < 0.001$ by ANOVA). Because of the high coefficient of variance, however, the differences between the estrogen/levormeloxifene groups and their L-NAME-treated counterparts did not reach statistical significance. Microscopically, intimal lesions appeared as raised focal areas between the endothelial cells and the internal elastic lamina, the predominating cell types being macrophage-derived foam cells and vascular smooth muscle cells.

Vascular reactivity

Aorta. Precontractions of aortic rings induced by 4×10^{-7} M phenylephrine were not significantly altered by treatment with L-NAME in either dose (L-NAME 160 group, 1.53 ± 0.09 ; L-NAME 320 group, 1.41 ± 0.07 vs. placebo group, 1.27 ± 0.08). Aortic rings in the L-NAME 160 group showed significantly less maximal relaxation in response to acetylcholine than those in the placebo group ($12 \pm 1\%$ vs. $42 \pm 4\%$, $P < 0.05$) (Fig. 3, top). No additional reduction of the maximal relaxation response was seen in the L-NAME 320 group ($10 \pm 1\%$). Relaxations caused by sodium nitroprusside were not significantly

different between groups, and thus were not altered by L-NAME treatment (Fig. 3, bottom).

Coronary artery. Virtually identical results for vascular reactivity were seen in the septal coronary artery (Fig. 4, top and bottom), except that the two L-NAME-treated groups were significantly more sensitive to the vasodilatory effect of sodium nitroprusside than was the placebo group ($P < 0.05$).

Discussion

This study confirms our previous findings that estrogen and levormeloxifene have a strong antiatherogenic effect mediated independently of changes in plasma lipids, and that this effect

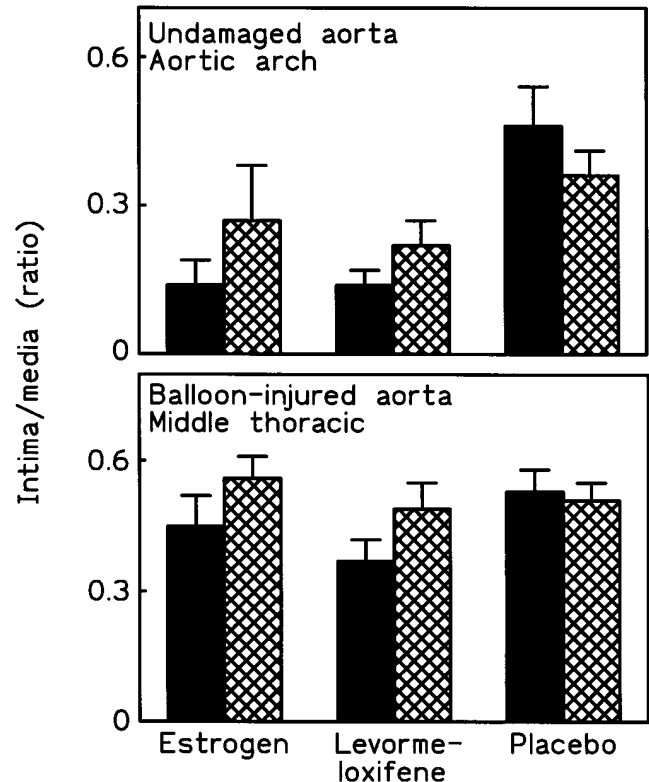


Figure 2. Intima/media ratio of the aortic arch (undamaged) and the middle thoracic aorta (balloon-injured) of the six groups. The data were well correlated with those for aortic cholesterol. All values are mean \pm SEM. Black bars, -L-NAME; hatched bars, +L-NAME.

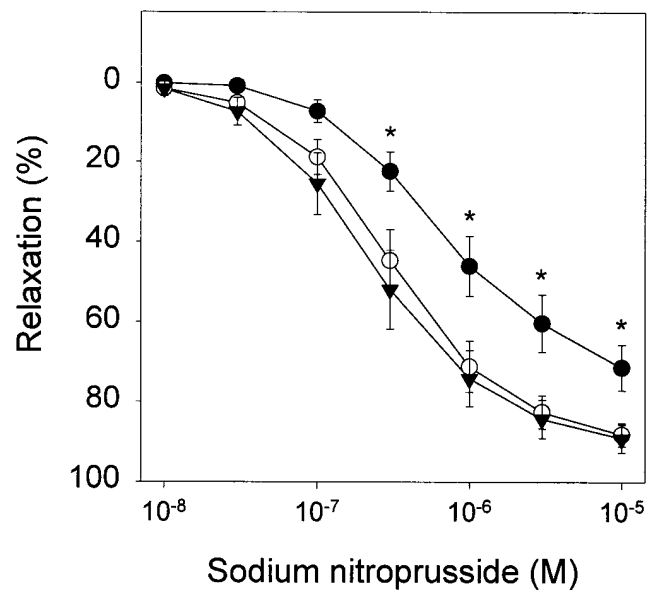
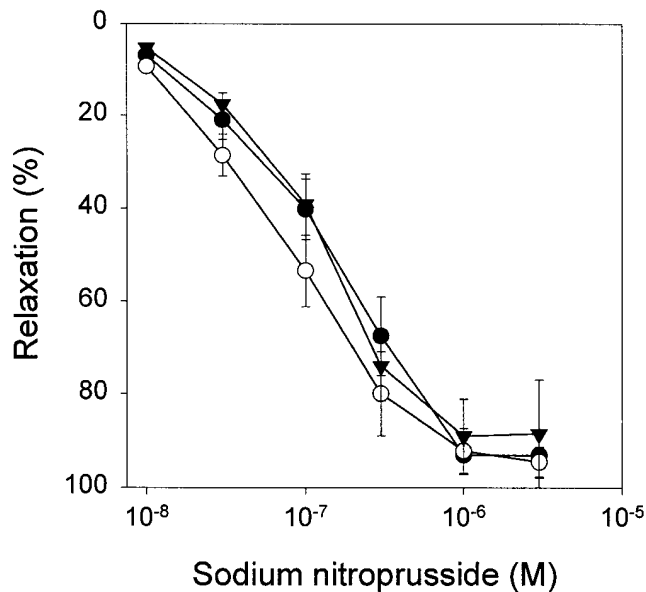
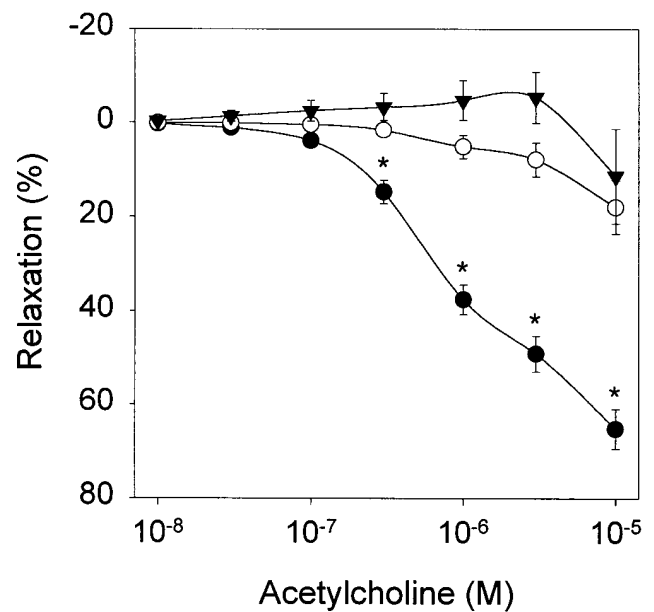
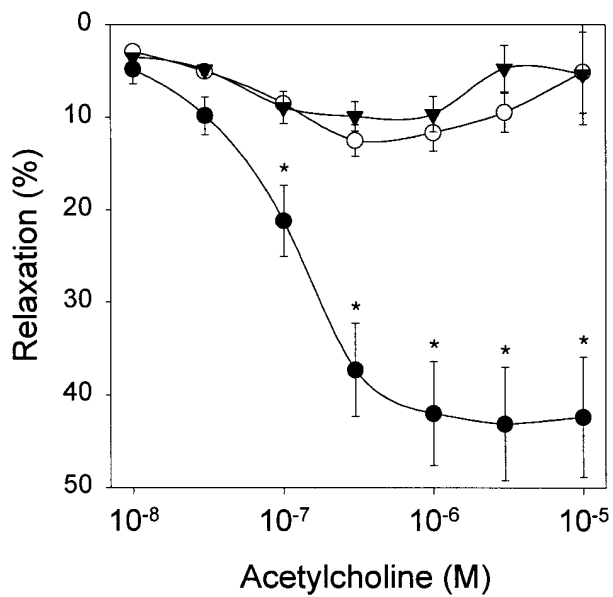


Figure 3. Effect of oral administration of L-NAME on acetylcholine- and sodium nitroprusside-induced relaxation in aortic rings from normocholesterolemic rabbits. 160 $\mu\text{g/ml}$ L-NAME and 320 $\mu\text{g/ml}$ L-NAME greatly and similarly inhibited the acetylcholine-induced relaxation response (*top*), whereas they had no effect on the relaxation response induced by sodium nitroprusside (*bottom*). The degree of relaxation is expressed as a percentage of the contraction induced by 4×10^{-7} M phenylephrine. All values are mean \pm SEM. * $P < 0.05$ compared with the L-NAME groups (Student's *t* test). Filled circles, placebo; open circles, L-NAME (160 $\mu\text{g/ml}$); filled triangles, L-NAME (320 $\mu\text{g/ml}$).

Figure 4. Effect of oral administration of L-NAME on acetylcholine- and sodium nitroprusside-induced relaxation in coronary artery rings from normocholesterolemic rabbits. The findings were similar to those obtained for the aortic rings, except that the sensitivity to sodium nitroprusside was significantly enhanced (*bottom*). The degree of relaxation is expressed as a percentage of the contraction induced by 30 mM potassium. All values are mean \pm SEM. * $P < 0.05$ compared with the L-NAME groups (Student's *t* test). Filled circles, placebo; open circles, L-NAME (160 $\mu\text{g/ml}$); filled triangles, L-NAME (320 $\mu\text{g/ml}$).

is abolished after mechanical removal of the endothelium by balloon catheter injury (22, 23).² More importantly, this study suggests that the administration of L-NAME, an inhibitor of endothelial NO synthesis, significantly attenuates the anti-

atherogenic effect of estrogen, and tends to attenuate the effect of levormeloxifene.

Findings from *in vitro* and *in vivo* studies suggest that 17β -estradiol upregulates the transcription of constitutive NOS in

the endothelium, and that this effect is mediated via the estrogen receptor (16–18). The constitutive release of NO from the endothelium includes a continuous basal release and a stimulated release that can be mediated by acetylcholine, other endothelium-dependent vasodilators, and mechanical forces such as shear stress (28). Estrogen treatment enhances endothelium-dependent relaxation to acetylcholine in nonatherosclerotic femoral arteries of rabbits (29), and attenuates acetylcholine-induced constriction in atherosclerotic coronary arteries of cynomolgus monkeys (11, 12). These findings have been confirmed in the coronary arteries of female (13, 14) but not male (15) human subjects, and are in good agreement with the suggestion of an estrogen receptor-mediated increase in endothelial constitutive NOS formation. Since the improvement by estrogen in acetylcholine-mediated relaxation is present within as little as 15 min after estrogen administration, however, other nontranscriptional, possibly non-NO-mediated effects of estrogen may be involved. Estrogen treatment may also affect the basal release of NO, since the basal release of NO from endothelium-intact aortic rings is greater in female rabbits than in male rabbits, a difference that is abolished by ovariectomy (21). It has been hypothesized that an increase in the continuous exposure of the arterial wall to NO, which may be an endogenous antiatherogenic molecule, is involved in the mechanism by which estrogen inhibits atherogenesis independently of changes in plasma lipids (18, 21). The present study adds support to this hypothesis, since the plasma lipid-independent antiatherogenic effect of estrogen was significantly attenuated by inhibition of arterial NO synthesis in cholesterol-fed rabbits.

Active vascular concentrations of L-NAME delivered in the drinking water were demonstrable in our short-term study by showing that endothelium-dependent relaxation of isolated aorta and coronary artery in response to acetylcholine was significantly inhibited. A similar inhibition of endothelium-dependent relaxation was observed in rabbits treated with estrogen and levormeloxifene (data not shown). In the aortic rings, endothelium-independent relaxation in response to sodium nitroprusside was not affected by L-NAME treatment, whereas in the coronary artery rings, the sensitivity to sodium nitroprusside was significantly enhanced. An increased sensitivity to nitrovasodilators after removal of endothelium or treatment with inhibitors of endothelial NO synthesis has previously been described *in vivo* and *in vitro*, and may be due to a specific supersensitivity to nitrovasodilators at the level of the soluble guanylate cyclase (30). In this study, the rabbits used for vascular reactivity measurements were treated with L-NAME for only 1 wk, and were not hypercholesterolemic. As similar results have been demonstrated in normocholesterolemic and hypercholesterolemic rabbits treated with L-NAME for 8 and 12 wk (31), however, it is likely that endothelium-derived NO synthesis was similarly reduced in our main study.

It has been reported previously that L-NAME elevates the blood pressure in rats (32, 33) and rabbits (34), but in those studies the NOS inhibitor was administered as a bolus intravenous injection. In our study and other studies using an oral/subcutaneous long-term administration of L-NAME (31, 35), blood pressure was not altered by treatment, possibly because this method maintains lower but more stable L-NAME concentrations.

The maintenance of plasma cholesterol at the same level in all the rabbits by individualized cholesterol feeding circum-

vented potential changes in plasma cholesterol by estrogen, levormeloxifene, or L-NAME, and made it possible exclusively to study plasma lipid-independent effects of these compounds. The increased amount of dietary cholesterol in the two estrogen/levormeloxifene groups compared to the placebo/placebo+L-NAME group indicates that estrogen and levormeloxifene treatment would have significantly decreased plasma cholesterol levels, had the rabbits not been cholesterol-clamped. The distribution of cholesterol between lipoprotein fractions was not grossly altered by estrogen, levormeloxifene, or L-NAME treatment.

The finding that L-NAME did not influence atherogenesis by itself in any of the four aortic sites (when compared with placebo rabbits not treated with L-NAME) is not consistent with those of two previous studies, in which administration of L-NAME significantly increased the severity of intimal lesions in cholesterol-fed rabbits (31, 35). The reason for this discrepancy is not known, but it may be related to the fact that the placebo animals in our experiment were severely affected by atherosclerosis, compared with those of the above-mentioned studies. A recent regression study in cholesterol-fed rabbits suggests that oral administration of the NO donor L-arginine can restore NO activity only until a certain point in atherogenesis, after which NOS is probably no longer able to convert L-arginine to NO (36). Such a deterioration of endothelial NOS activity by atherosclerosis may explain the observation that L-NAME had no effect on atherogenesis in aortas with severe atherosclerosis.

Thus, in our experiment L-NAME did not alter plasma cholesterol, plasma lipoprotein distribution, arterial blood pressure, or atherogenesis, yet it significantly attenuated the plasma lipid-independent antiatherogenic effect of estrogen. L-NAME at a dose of 320 $\mu\text{g}/\text{ml}$ did not further inhibit endothelium-dependent relaxation in response to acetylcholine in our short-term study, suggesting that a maximal inhibition of endothelium-derived NO synthesis was achieved with the current dose of 160 $\mu\text{g}/\text{ml}$. Still, the aortic accumulation of cholesterol was nonsignificantly ($P = 0.08$) lower in the estrogen group treated with L-NAME than in the placebo group treated with L-NAME. This suggests that mechanisms other than NO are involved in the mechanism by which estrogen inhibits atherogenesis directly in the arterial wall. These mechanisms could involve effects of estrogen on smooth muscle cell proliferation (37), monocyte-endothelial cell interaction (38), chemokines (39), or lipid peroxidation (40).

The addition of L-NAME to estrogen-treated rabbits also resulted in a worsening of atherogenesis in the balloon-injured site, and as estrogen had no effect, this resulted in a net atherogenic effect of the combination of estrogen and L-NAME. Currently, we have no explanations for these findings, but they may be due to an interaction between estrogen and NO generated either from regenerating endothelial cells or from nonendothelial cells, and they may be mediated via mechanisms other than those working in undamaged aorta.

The mechanism(s) by which NO, at least in part, mediates the antiatherogenic effect of estrogen in the undamaged aorta was not elucidated by this study. NO is known to inhibit platelet adhesion and aggregation, and also inhibits the proliferation of smooth muscle cells (41–43). Moreover, NO may decrease endothelial adhesiveness for monocytes and the expression of monocyte chemotactic protein (44); probably by virtue of its capability to reduce intracellular oxidative stress,

thereby suppressing oxidant-responsive genes encoding adhesion molecules and chemokines (45–47). Similar regulatory effects of estrogen on the above processes have been described previously (37–40).

Levormeloxifene had, like estrogen, a significant plasma lipid-independent antiatherogenic effect in the undamaged aorta, but no effect in the balloon-injured site. This is consistent with our previous findings.² The antiatherogenic effect of levormeloxifene tended to be inhibited by the addition of L-NAME, providing further evidence that levormeloxifene acts on the artery through the same mechanisms as estrogen. In contrast to estrogen, however, levormeloxifene has no noticeable effect on reproductive tissue.²

In conclusion, this study adds support to the hypothesis that part of the direct antiatherogenic effect of estrogen on the arterial wall is mediated through its effect on endothelial NO in cholesterol-fed rabbits. We therefore suggest that NO is involved in both the hemodynamic and antiatherogenic effects of estrogen.

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