In vitro lipid peroxidation of LDL from postmenopausal cynomolgus macaques treated with female hormones

Dawn C. Schwenke,[1,*] Janice D. Wagner,[†] and Michael R. Adams[†]

Department of Pathology* and the Section of Comparative Medicine,[†] Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1072

Abstract Premenopausal women and postmenopausal women given estrogen are protected from cardiovascular diseases compared with men. Previous studies investigated whether estrogen treatment protects low density lipoprotein (LDL) from in vitro oxidation as a potential mechanistic explanation for the beneficial effect of estrogen. Results of these studies are mixed, and very few studies considered aspects of LDL that influence LDL oxidation. This study investigated whether treating postmenopausal female cynomolgus macaques with conjugated equine estrogens (CEE), medroxyprogesterone acetate (MPA), CEE + MPA, or tamoxifen, a mixed estrogen receptor agonist/antagonist, would protect LDL from in vitro oxidation. LDL was isolated from monkeys fed an atherogenic diet for 12 weeks or the same diet with CEE, MPA, CEE + MPA, or tamoxifen added at levels equivalent (on a caloric basis) to those given to women. LDL was subjected to Cu^{2+} (3 μmol/L) or 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH, 1 mmol/L) and LDL oxidation was determined by the lag time before rapid formation of conjugated dienes and by the maximal rate of conjugated diene formation (propagation rate). Lag times and propagation rates were not affected by treatment. Lag times for Cu^{2+} oxidation were related to LDL tocopherol while lag times for AAPH oxidation were related to high density lipoprotein (HDL) cholesterol and to LDL molecular weight. Multivariate analysis showed that LDL α- and γ-tocopherol together could explain 27% of the variation in Cu^{2+} mediated lag time ($P < 0.005$) among animals while HDL cholesterol, LDL γ-tocopherol, and LDL molecular weight combined could explain 40% of the variation in AAPH-mediated lag time ($P = 0.0006$) among animals. After adjustment for these predictors, LDL lag times were not affected by treatment. In conclusion, in monkeys treated with female hormones, multiple factors influence in vitro low density lipoprotein (LDL) oxidation; future work will be needed to determine whether estrogen alters oxidation of LDL in the artery.—Schwenke, D. C., J. D. Wagner, and M. R. Adams. **In vitro lipid peroxidation of LDL from postmenopausal cynomolgus macaques treated with female hormones. *J. Lipid Res.* 1999.** 40: **235–244.**

Supplementary key words tamoxifen • conjugated equine estrogen • LDL oxidation • lag time • lipid peroxidation • alpha tocopherol • gamma tocopherol • vitamin E • antioxidant • oxidation

Premenopausal women are relatively protected from cardiovascular disease compared with men (1, 2). In contrast, postmenopausal women experience risk of cardiovascular disease that is similar to that of men of the same age (1, 3). Population-based studies (4, 5) suggest that treatment with estrogen (with or without progestin) can greatly reduce risk of cardiovascular disease in postmenopausal women. Estrogen could reduce clinical cardiovascular disease by improving vascular reactivity (6). Also, studies in experimental animals have shown that estrogen reduces atherosclerosis, the underlying cause of cardiovascular disease (7), by mechanism(s) that are independent of effects on lipids, lipoproteins, and apoproteins (8–11). Similarly, a study in women showed that estrogen replacement therapy was associated with reduced progression of carotid artery intima-media thickness, an early measure of atherosclerosis, and that this effect was independent of lipoproteins (12). Tamoxifen, a mixed estrogen receptor agonist/antagonist (13), reduces the rate of death due to acute myocardial infarction relatively more than expected based on changes in lipoproteins (14).

Evidence suggests that intra-arterial oxidation of low density lipoprotein may play a role in atherogenesis (7, 15). Eleven studies with data for postmenopausal women (16–26) investigated the influence of estrogen (with or without progestin) on susceptibility of LDL to in vitro lipid peroxidation. The results of these studies are inconsistent. However, typically studies with transdermal estrogen delivery (with or without oral progestin) found that estrogen increased resistance of LDL to oxidation (16, 17, 25, 26) while most studies with oral estrogen treatment (with or without progestin) found that estrogen had no effect on resistance of LDL to oxidation (18, 20–

Abbreviations: MPA, medroxyprogesterone acetate; CEE, conjugated equine estrogens; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; PPACK, d-phenylalanyl-l-prolyl-arginine chloromethyl ketone; EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline.

[1]To whom correspondence should be addressed.

23, 26). One study reported that tamoxifen increased resistance of LDL to oxidation (18). Data suggest that estrogens could increase LDL vitamin E (27) and reduce average LDL molecular size (11, 28–30). LDL α-tocopherol (31–34) and LDL molecular size (31, 35–38) influence susceptibility of LDL to in vitro lipid peroxidation. Yet only a few of the studies that investigated the effect of estrogen on resistance of LDL to oxidation considered either of these factors (21, 23, 39) and none of these studies explicitly investigated the effect of aspects of LDL character as determinants of resistance of LDL to in vitro lipid peroxidation.

In this study we investigated the influence of in vivo treatment of surgically postmenopausal female cynomolgus macaques with conjugated equine estrogens (CEE), medroxyprogesterone acetate (MPA), or both, commonly used forms of hormone replacement in postmenopausal women (18, 21, 22, 26), or tamoxifen, on resistance of isolated LDL to in vitro lipid peroxidation. In this study in which all experimental subjects received diets with identical vitamin E content, we found that neither the individual female hormones, the hormone combination, nor tamoxifen influenced resistance of LDL to in vitro lipid peroxidation. However, we did find that a significant portion of the variance for resistance of LDL to in vitro lipid peroxidation could be accounted for by differences in LDL α- and γ-tocopherol, LDL molecular weight, and HDL cholesterol. These results suggest that oral CEE has little, if any, direct effect on resistance of LDL to in vitro lipid peroxidation.

METHODS

Animals

Thirty eight feral adult female cynomolgus monkeys (*Macaca fascicularis*) imported from Indonesia (CV Primates, Bogor, Indonesia) were studied. These animals were a subset of the animals used for other studies (28, 40). After quarantine and challenge with atherogenic diet, monkeys were stratified into five groups with similar plasma and HDL cholesterol concentrations (28, 40). Thereafter, monkeys were fed monkey chow, to allow plasma cholesterol concentrations to return to baseline values, and subjected to bilateral ovariectomy as described (28, 40). 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 Animal Care and Use Committee of the Wake Forest University School of Medicine.

In vivo hormone treatment

During the period of hormone treatment, all monkeys were fed a moderately atherogenic diet with 40% of calories as fat and containing 0.28 mg cholesterol/kcal (28, 40). The sources of fat in the diet were lard, butter, beef tallow, dried egg yolk, and safflower oil. Of the dietary fatty acids, 46% were saturated, 43% were monounsaturated, and 11% were polyunsaturated. All hormones were incorporated into the diet. The treatment groups were as follows: Group 1, untreated control (ovariectomized hormone deficient, n = 8) monkeys; Group 2, monkeys treated with CEE (0.04 mg/kg per day, n = 8, Premarin, Wyeth-Ayerst, Radnor, PA); Group 3, monkeys treated with MPA (0.17 mg/kg per day, n = 8, Cycrin, Wyeth-Ayerst); Group 4, monkeys given both CEE and MPA at the same levels given to Groups 2 and 3 (n = 8); Group 5, monkeys given tamoxifen citrate (1.33 mg/kg per day, n = 8) (Nolvadex, ICI Pharma, Wilmington, DE). All hormone doses were calculated by scaling usual daily doses for women (0.625 mg CEE, 2.5 mg MPA, 20 mg tamoxifen) to the relative caloric needs of the monkeys (11, 28, 40) and assuming that an average woman consumes 1800 kcal/day. The diet contained 4.18 mg α-tocopherol and 0.4 mg other tocopherols per 100 g. This is equivalent to 16.8 mg α-tocopherol and 1.6 mg other tocopherols per 1800 kcal, about twice the recommended dietary intake for women (41). After 12 weeks of treatment, and just before killing as required by other studies (28, 40), a 30-ml blood sample was collected for measurement of lipids and separation of plasma lipoproteins.

Plasma lipids, lipoproteins, and hormones

Concentrations of cholesterol in plasma and in high density lipoproteins (HDL) and triglycerides in plasma were determined at the end of the 12-week period of treatment (42). These measurements were done in a lipid analytical laboratory at the Wake Forest University School of Medicine which is in full standardization with the Centers for Disease Control–National Heart, Lung, and Blood Institute Lipid Standardization Program. NonHDL cholesterol (very low density plus intermediate density plus low density lipoproteins) was calculated as the difference between plasma and HDL cholesterol concentrations. LDL molecular weight was determined by column chromatography after 9 weeks of treatment (28, 40). Plasma concentrations of estradiol and estrone were measured by radioimmunoassay as described previously (43) in samples collected after an overnight fast and after 4 and 7 weeks of treatment (28, 40). In some cases, negative values were obtained for estradiol and/or estrone. In these cases, values of zero were assumed. The 4-week and 7-week values of these hormones were averaged for use in analyses described below.

Isolation of LDL for oxidation studies

LDL ($1.020 < d < 1.060$ g/ml) used in these studies was isolated by differential centrifugation (44, 45) from fresh plasma of individual monkeys. Blood was collected into a cocktail of 1 μm d-phenylalanyl-l-prolyl-arginine chloromethyl ketone (PPACK) and 25 kallikrein inhibitory units/ml aprotinin to limit degradation of apolipoprotein B (apoB) by proteolysis and disodium EDTA at 2.7 mmol/L to prevent oxidation (44, 45). Plasma was immediately separated from red cells. The plasma was mixed with 0.5 mmol/L of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Isolated LDL was dialyzed against 3–4 changes of 200 volumes of 20 mol/L phosphate-buffered saline (0.15 mol/L) containing 2 mmol/L EDTA (PBS+EDTA) (44, 45) during a period of 2–4 days.

Composition of LDL

The protein concentration of LDL was determined with the Folin phenol reagent as described (44, 45). LDL was characterized by determining the content of endogenous antioxidants. Antioxidants (α- and γ-tocopherol, carotenes, and lycopene) in aliquots of LDL (about 1.5 mg total lipid) were quantitated in the presence of tocol as an internal standard for tocopherols (46) and retinol palmitate as an internal standard for carotenes and lycopene (47). These measurements were done by the method of Elinder and Walldius (47) with the mobile phase modified to acetonitrile–tetrahydrofuran–methanol–1% ammonium acetate (684:220:68:28) as described previously for antioxidants in plasma (48). These assays were conducted in a core laboratory of the Wake Forest University School of Medicine Comprehensive Cancer Center.

Susceptibility of LDL to in vitro lipid peroxidation

About 24 h before initiating measurements of susceptibility to lipid peroxidation, LDL was dialyzed against three 200 volume changes of 10 mmol/L phosphate-buffered saline (0.15 mol/L) free of EDTA (PBS) which was continuously sparged with nitrogen. Susceptibility of LDL to lipid peroxidation was assessed by evaluating the formation of conjugated dienes while the LDL was exposed to the oxidizing stress provided by 3 μmol/L Cu^{2+} or 1 mmol/L 2,2′- azobis(2-amidinopropane) dihydrochloride (AAPH). LDL (50 μg protein/ml final concentration) was incubated at 37°C in a cuvette with either of the oxidizing stressors in the presence of PBS. The formation of conjugated dienes was followed by monitoring the absorbance at 234 nm continuously for up to 24 h (49, 50) in a Uvikon 940 spectrophotometer equipped with a thermostatted cell changer holding 12 cuvettes. LDL samples isolated from one monkey of each group were tested simultaneously in duplicate for susceptibility to lipid peroxidation. The assay was repeated on the next day to accumulate four replicate assays of each LDL sample. Cu^{2+} mediated oxidations were done first. Immediately afterwards, the remaining LDL samples were supplemented with EDTA (2.7 mmol/L final concentration). AAPH-mediated oxidations were done during the following 2–5 days to accumulate four replicate assays for each LDL sample. Conjugated diene formation during the exposure to oxidizing stress was calculated from the increase in absorption at 234 nm above the initial value and the molar absorptivity of conjugated dienes (29,500 liter $mol^{-1}cm^{-1}$ (50, 51). We determined two indices of the susceptibility of LDL to oxidation: the lag time before conjugated dienes began to increase significantly (51) and the maximum rate of conjugated diene formation (propagation rate) (50, 51).

In other studies, LDL isolated from other postmenopausal female cynomolgus monkeys fed a similar atherogenic diet but not treated with hormones were supplemented in vitro with 17β-estradiol or tamoxifen during the assessment of susceptibility to lipid peroxidation. Exogenous hormones (0.25–15 μmol/L final concentration) in absolute ethanol (0.5% final concentration) were added to LDL in PBS. Control LDL samples contained the same concentration of ethanol but no hormone.

Statistical methods

Data for the five groups of monkeys were analyzed by analysis of variance (52). The fraction of the variation in lag times and propagation rates for oxidation of LDL explained by different plasma parameters was investigated by linear regression (52). The plasma parameters considered were LDL α- and γ-tocopherol, LDL molecular weight, plasma concentrations of estradiol, estrone, cholesterol, and triglyceride, and HDL cholesterol and nonHDL cholesterol. LDL α-tocopherol and LDL molecular weight were considered because previous studies showed that LDL α-tocopherol (31–34) and LDL molecular size (31, 35–38) influenced susceptibility of LDL to in vitro lipid peroxidation. Because LDL molecular weight was measured at 9 weeks of treatment (28, 40), rather than after 12 weeks when LDL was isolated for studies of oxidizability, we included plasma (53) and HDL (54, 55) cholesterol and plasma triglyceride (53–56) concentrations measured at the time of the oxidation study as surrogates that are related to LDL molecular size and/or weight. NonHDL cholesterol and γ-tocopherol were considered for comparison with HDL cholesterol and α-tocopherol, respectively. Differences among groups for the associations of LDL lag times and LDL propagation rates with the various plasma parameters were investigated by comparing the total regression sums of squares for all groups analyzed together with the total sums of squares for individual groups analyzed separately using Fisher's F-test (57).

Stepwise multivariate regression was used to select the set of parameters best accounting for LDL lag times and propagation rates. Additional predictive parameters were added to the best univariate model only if their inclusion significantly increased the predictive ability of the model. These comparisons were done with Fisher's F-test (57). Because HDL, nonHDL, and plasma cholesterol are linearly dependent, these analyses were done twice, once with all the parameters except plasma cholesterol and a second time with plasma cholesterol but excluding HDL and nonHDL cholesterol. A P value of less than 0.05 was considered significant.

RESULTS

Plasma lipids and lipoproteins

Plasma cholesterol concentrations averaged from 5.33 ± 0.74 to 6.97 ± 0.58 mmol/L with no difference among the five groups (**Table 1**). Similarly, triglyceride concentrations averaged 0.30 ± 0.06 to 0.73 ± 0.18 mmol/L with no difference among the five groups. HDL and nonHDL cholesterol concentrations averaged 0.74 ± 0.07 to 1.04 ± 0.11 and 4.44 ± 0.97 to 6.06 ± 0.67 mmol/L, respectively, with no difference among the different treatment groups.

Plasma hormone concentrations

Plasma concentrations of estradiol and estrone differed significantly among treatment groups ($P < 0.0004$ and $P < 0.0017$, respectively, **Table 2**). Oral dosing with CEE increased plasma concentrations of both estradiol ($P = 0.0000$) and estrone ($P < 0.0002$) while neither MPA nor tamoxifen influenced plasma concentrations of estradiol or estrone.

TABLE 1. Lipids and lipoproteins

Treatment Group	Plasma Triglyceride	Plasma Cholesterol	HDL Cholesterol	NonHDL Cholesterol	LDL Molar Mass
		mmol/L			*g/μmol*
Control	0.66 ± 0.22	6.48 ± 0.57	1.00 ± 0.13	5.48 ± 0.55	4.44 ± 0.09
MPA	0.30 ± 0.06	6.97 ± 0.58	0.91 ± 0.14	6.06 ± 0.67	4.70 ± 0.20
CEE	0.73 ± 0.18	5.93 ± 0.52	0.74 ± 0.07	5.19 ± 0.86	4.35 ± 0.19
CEE + MPA	0.59 ± 0.15	5.33 ± 0.74	0.89 ± 0.17	4.44 ± 0.97	4.10 ± 0.11
Tamoxifen	0.72 ± 0.19	5.55 ± 0.57	1.04 ± 0.11	4.51 ± 0.63	4.70 ± 0.19
ANOVA	0.36	0.30	0.55	0.36	0.071

HDL, high density lipoproteins; nonHDL, very low density + intermediate density + low density lipoproteins; ANOVA, analysis of variance. Values are mean ± SEM for 7 (CEE group, CEE + MPA group) or 8 monkeys (all other groups). To obtain cholesterol and triglyceride concentrations in mg/dl, multiply values in mmol/L by 38.7 and 88.5, respectively.

TABLE 2. Plasma hormone concentrations

Treatment Group	Estradiol	Estrone
	pmol/L	
Control	8 ± 5	9 ± 8
MPA	12 ± 5	3 ± 3
CEE	209 ± 58	126 ± 35
CEE + MPA	173 ± 33	207 ± 82
Tamoxifen	57 ± 48	22 ± 20
ANOVA	0.0004	0.0017
CEE[a]	0.0000	0.0002
MPA[b]	0.66	0.33
Tamoxifen[c]	0.33	0.80

Values are mean ± SEM of the average of measurements made after 4 and 7 weeks of treatment. For CEE and CEE + MPA groups, n = 7; for all other groups, n = 8. ANOVA, analysis of variance.

[a]Control and MPA groups contrasted with CEE and CEE + MPA groups.

[b]Control and CEE groups contrasted with MPA and CEE + MPA groups.

[c]Control group compared with tamoxifen group.

TABLE 4. Influence of in vivo treatment of surgically post-menopausal female cynomolgus monkeys with MPA, CEE, and tamoxifen on susceptibility of LDL to lipid peroxidation in vitro

Treatment Group	Lag Time		Propagation Rate	
	Cu^{2+}	AAPH	Cu^{2+}	AAPH
	min		*nmol conjugated diene/min/mg LDL protein*	
Control	110 ± 6	52 ± 4	4.16 ± 0.42	0.80 ± 0.06
MPA	118 ± 6	53 ± 4	3.69 ± 0.15	0.73 ± 0.04
CEE	112 ± 6	55 ± 3	4.05 ± 0.19	0.83 ± 0.05
CEE + MPA	110 ± 8	55 ± 4	3.92 ± 0.19	0.74 ± 0.04
Tamoxifen	111 ± 8	56 ± 5	3.96 ± 0.21	0.82 ± 0.04
ANOVA	0.92	0.93	0.75	0.47

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride. Values are mean ± SEM for 7 (CEE group, CEE + MPA group) or 8 monkeys (all other groups). Lag times and propagation rates were determined as described in Methods for LDL at 37°C exposed to 3 μmol/L Cu^{2+} or 1 mmol/L AAPH.

Endogenous antioxidants in LDL

Per mg protein, isolated LDL particles contained an average of 2.74 ± 0.29 to 3.55 ± 0.32 μg α-tocopherol and 0.094 ± 0.007 to 0.143 ± 0.027 μg γ-tocopherol, with no difference among the treatment groups (**Table 3**). Neither β- nor α-carotene nor lycopene could be detected in these LDL samples. Assuming that apoB accounts for all of the protein in LDL and using the reported molecular weight of apoB (58, 59), we calculated the number of molecules of α- and γ-tocopherol associated with LDL particles. Molecules of α- and γ-tocopherol per LDL particle ranged from 3.27 ± 0.20 to 4.22 ± 0.38 and 0.116 ± 0.008 to 0.176 ± 0.034, respectively, with no difference among groups (Table 3).

Influence of in vivo supplementation with female hormones or tamoxifen on susceptibility of LDL to in vitro oxidation

Isolated LDL were subjected to oxidation mediated by Cu^{2+} (3 μmol/L) and, in separate studies, oxidation mediated by the azo initiator AAPH (1 mmol/L). Lag times ranged from 110 ± 6 to 118 ± 6 min after exposure to Cu^{2+} and from 52 ± 4 to 56 ± 5 min after exposure to AAPH with no difference among groups for either oxidizing stress (**Table 4**). Propagation rates ranged from 3.69 ± 0.15 to 4.16 ± 0.42 nmol/conjugated diene per min per mg LDL protein for LDL exposed to Cu^{2+} and 0.73 ± 0.04 to 0.83 ± 0.05 nmol/conjugated diene per min per mg LDL protein for LDL exposed to AAPH with no difference among groups for either oxidizing stress.

Association of LDL lag times and propagation rates with LDL antioxidants, plasma hormones, lipids and lipoproteins

LDL lag times after exposure to Cu^{2+} were positively associated with LDL γ-tocopherol ($r = 0.39$, $P < 0.02$, **Table 5**) and inversely associated with LDL α-tocopherol ($r = -0.31$, $P < 0.06$). LDL lag times after exposure to AAPH were positively associated with HDL cholesterol ($r = 0.44$, $P < 0.006$) and inversely with LDL molecular weight ($r = -0.044$, $P < 0.006$). For monkeys treated with CEE (with or without MPA), LDL lag times after exposure to Cu^{2+} and AAPH were inversely associated with plasma concentrations of estradiol ($r = -0.56$, $P < 0.04$ and $r = -0.52$, $P < 0.06$, respectively). Propagation rates for LDL exposed to Cu^{2+} or AAPH were not significantly associated with any

TABLE 3. Endogenous antioxidants in LDL from surgically post-menopausal female cynomolgus monkeys treated in vivo with MPA, CEE, and tamoxifen

Treatment Group	μg/mg LDL Protein		Molecules/LDL Particle	
	α-Tocopherol	γ-Tocopherol	α-Tocopherol	γ-Tocopherol
Control	2.74 ± 0.29	0.094 ± 0.007	3.27 ± 0.35	0.116 ± 0.008
MPA	2.74 ± 0.16	0.133 ± 0.015	3.27 ± 0.20	0.164 ± 0.019
CEE	3.18 ± 0.48	0.143 ± 0.027	3.79 ± 0.57	0.176 ± 0.034
CEE + MPA	3.10 ± 0.59	0.116 ± 0.027	3.69 ± 0.70	0.143 ± 0.034
Tamoxifen	3.55 ± 0.32	0.127 ± 0.016	4.22 ± 0.38	0.156 ± 0.020
ANOVA	0.28	0.31	0.28	0.31

Values are mean ± SEM for 7 (CEE group, CEE + MPA group) or 8 monkeys (all other groups). Neither α- nor β-carotene nor lycopene could be detected in any LDL sample. Molecules of α- and γ-tocopherol were calculated from literature values for the molecular weight of apolipoprotein B (58, 59) and assuming that apolipoprotein B accounted for all protein in LDL.

TABLE 5. Association of LDL lag times and propagation rates with LDL antioxidants, plasma hormones, lipids and lipoproteins for surgically post-menopausal female cynomolgus monkeys treated with MPA, CEE, and tamoxifen

	Cu^{2+} Oxidation		AAPH Oxidation	
Parameter	Lag Time	Prop. Rate	Lag Time	Prop. Rate
α-T/LDL	−0.31[a]	0.28	−0.03	0.18
γ-T/LDL	0.39[b]	−0.18	0.24	−0.08
HDL cholesterol	−0.26	0.12	0.44[c]	0.06
NonHDL cholesterol	0.21	−0.05	−0.07	−0.19
Triglyceride	−0.17	0.11	−0.26	−0.04
LDL MW	−0.03	0.06	−0.44[c]	0.09
Estradiol	−0.25	0.08	−0.18	0.02
	−0.56[e,d]	0.24[e]	−0.52[a,e]	0.01[e]
Estrone	−0.17	−0.03	−0.06	−0.01
	−0.28[e]	−0.14[e]	−0.20[e]	−0.03[e]

α-T/LDL and γ-T/LDL, molecules α- and γ-tocopherol, respectively, per LDL particle. LDL MW, LDL molecular weight. Correlations were determined for all treatment groups considered together (n = 38) because there were no significant differences among groups for the nature of the association between any parameter and LDL lag time or propagation rate ($P \geq 0.2$ in all cases and typically >0.5).

[a] $P < 0.06$; [b] $P < 0.02$; [c] $P < 0.006$; [d] $P < 0.04$.

[e] Monkeys given CEE (n = 7) and those given CEE + MPA (n = 7).

of the plasma hormones, lipids, lipoproteins, or LDL antioxidants that were measured.

Multivariate models best explaining the variation in LDL lag time

Multiple linear regression showed that several plasma and LDL parameters contributed independently to explaining variation in LDL lag time among animals for both Cu^{2+}-mediated and AAPH-mediated oxidation (**Table 6**). For all treatment groups combined, LDL γ- and α-tocopherol each independently contributed significantly ($P < 0.05$) to variation in LDL lag time after exposure to Cu^{2+}, together accounting for 27% ($P = 0.0042$) of the variation in LDL lag time. For all treatment groups combined, HDL cholesterol ($P < 0.01$), LDL γ-tocopherol ($P < 0.02$), and LDL molecular weight ($P < 0.05$) each independently contributed significantly to variation in LDL lag time after exposure to AAPH, together accounting for 40% ($P = 0.0006$) of the variation in LDL lag time. There was no difference among treatment groups for the association of the above parameters with lag time for Cu^{2+} ($P = 0.51$) or AAPH-mediated oxidation ($P = 0.96$). The multivariate models predicted that a one standard deviation change in each of the predictive variables would alter lag times for Cu^{2+}- and AAPH-mediated oxidation by 6–7 and 3–4 min, respectively (6–7% and 6–8%, respectively) (Tables 4, 6). After adjustment for the predictors identified by multivariate analysis, lag times after Cu^{2+}- and AAPH-mediated oxidation did not differ among treatment groups (**Table 7**).

Comparison of susceptibility of LDL to lipid peroxidation initiated in vitro by Cu^{2+} and AAPH

Under our experimental conditions, lag times were about twice as long for Cu^{2+}-mediated oxidation as for AAPH-mediated oxidation (Table 4). In comparison, propagation rates were more than 4-fold as great for Cu^{2+}-mediated lipid peroxidation as for AAPH-mediated lipid peroxidation (Table 4). Lag times for Cu^{2+}- and AAPH-mediated lipid peroxidation were highly correlated ($r = 0.53$, $P = 0.0007$) as were propagation rates ($r = 0.68$, $P < 0.0001$), with no differences in these relationships for the different treatment groups ($P = 0.61$, $P = 0.53$, respectively).

TABLE 6. Multivariate models best explaining variation in LDL lag time for in vitro lipid peroxidation for LDL isolated from surgically post-menopausal female cynomolgus monkeys

Rank Order of Parameters Contributing to LDL Lag Time and Contribution to LDL Lag Time

Cu^{2+} Oxidation			AAPH Oxidation		
Rank	Parameter	Cum. SS[a]	Rank	Parameter	Cum. SS
1	γ-T/LDL[b]	0.150	1	HDL Chol[b]	0.196
2	α-T/LDL[c]	0.269	2	γ-T/LDL[c]	0.321
			3	LDL MW[d]	0.396
Total		($P = 0.0042$)[e]	Total		($P = 0.0006$)

Predicted Changes in LDL Lag Time for a 1 SD Change in Predictive Parameter

Cu^{2+} Oxidation			AAPH Oxidation		
Parameter	SD	Predicted Change in Minutes[f]	Parameter	SD	Predicted Change in Minutes
γ-T/LDL	0.0582	7.4 ± 2.6	HDL Chol	0.350	4.3 ± 1.6
α-T/LDL	0.995	−6.2 ± 2.6	γ-T/LDL	0.0582	3.6 ± 1.4
			LDL MW	0.479	−3.3 ± 1.5

[a] Cumulative fraction of total sum of squares explained by the parameter(s) listed. α-T/LDL, γ-T/LDL, molecules of α- and γ-tocopherol per LDL particle, calculated as described in Table 3. HDL Chol, HDL cholesterol; LDL MW, LDL molecular weight.

[b] $P < 0.01$; [c] $P < 0.025$; [d] $P < 0.05$: significance of the contribution of individual parameters to sums of squares.

[e] Significance of the entire set of parameters.

[f] Change in LDL lag time in minutes predicted by a 1 SD change in the parameter listed.

Influence of in vitro supplementation of LDL with female hormones

Figure 1 shows the effects of increasing concentrations of exogenous 17β-estradiol and tamoxifen on lag time

TABLE 7. Adjusted values for lag times for in vitro lipid peroxidation for LDL from surgically post-menopausal female cynomolgus monkeys treated in vivo with MPA, CEE, and tamoxifen

Treatment Group	Lag time	
	Cu^{2+}[a]	AAPH[b]
	min	
Control	112 ± 6	53 ± 3
MPA	113 ± 6	54 ± 3
CEE	109 ± 6	54 ± 4
CEE + MPA	111 ± 6	53 ± 4
Tamoxifen	114 ± 6	56 ± 3
ANCOVA	0.99	0.95

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride. ANCOVA, analysis of covariance. Values are mean ± SEM for 7 (CEE group, CEE + MPA group) or 8 monkeys (all other groups).

[a] Adjusted for differences in LDL α- and γ-tocopherol (Table 6).

[b] Adjusted for differences in LDL γ-tocopherol, LDL molecular weight, and HDL cholesterol (Table 6).

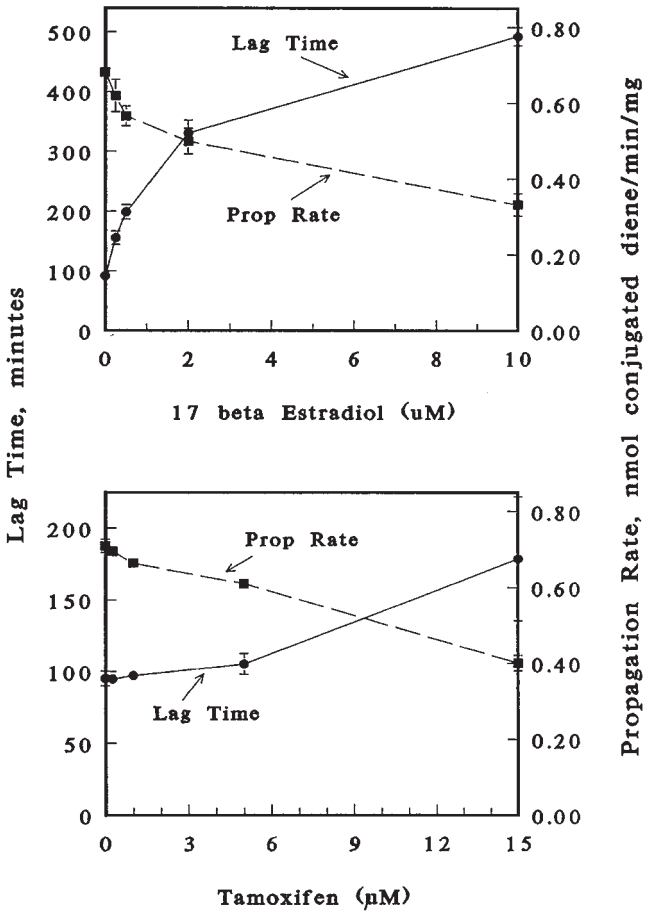

Fig. 1. Top, inhibition of AAPH-mediated oxidation of monkey LDL by in vitro addition of 17β-estradiol. Filled circles and solid line, lag time; filled squares and broken line, propagation rate. Values are mean ± SEM of 3–4 observations for each concentration of estradiol. Regression of lag time on estradiol concentration, $r = 0.92$, $P < 0.0001$; regression of lag time on log(estradiol concentration +1), $r = 0.98$, $P < 0.0001$. Regression of propagation rate on estradiol concentration, $r = -0.87$, $P < 0.0001$; regression of propagation rate on log(estradiol concentration +1), $r = -0.91$, $P < 0.0001$. Comparison of lag times for estradiol-supplemented LDL and unsupplemented LDL, $P < 0.004$ for all concentrations of estradiol greater than 0.25 μmol/L. Comparison of propagation rates for estradiol-supplemented and unsupplemented LDL, $P < 0.03$ for 0.5 μmol/L and $P < 0.008$ for all higher concentrations, Bonferroni correction for multiple comparisons. Bottom, inhibition of AAPH-mediated oxidation of monkey LDL by in vitro addition of tamoxifen. Filled circles and solid line, lag time; filled squares and broken line, propagation rate. Values are mean ± SEM of four observations for each concentration of tamoxifen. Regression of lag time on tamoxifen concentration, $r = 0.67$, $P = 0.001$. Regression of propagation rate on tamoxifen concentration, $r = -0.97$, $P < 0.0001$. No individual comparison of lag times for tamoxifen-supplemented LDL with unsupplemented LDL were significant. Comparison of propagation rates for tamoxifen-supplemented and unsupplemented LDL, $P < 0.02$ for 5 μmol/L and $P < 0.0002$ for 15 μmol/L tamoxifen, Bonferroni correction for multiple comparisons.

and propagation rate of LDL exposed to AAPH. Lag times of LDL oxidation were positively associated with estradiol concentrations ($r = 0.92$, $P < 0.0001$, Fig. 1, top) and even more closely associated with log(estradiol concentration +1) ($r = 0.98$, $P < 0.0001$). Propagation rates for LDL oxidation were inversely associated with estradiol concentrations ($r = -0.87$, $P < 0.0001$). The lowest concentration of 17β-estradiol at which LDL lag time was significantly prolonged was 0.25 μmol/L while the lowest concentration of 17β-estradiol at which LDL propagation rate was significantly reduced was 0.5 μmol/L (Fig. 1, top). LDL lag times were positively associated with tamoxifen concentration ($r = 0.67$, $P = 0.001$, Fig. 1, bottom), but none of the individual comparisons between unsupplemented LDL and tamoxifen-supplemented LDL were significant for LDL lag time. Propagation rates for LDL oxidation were inversely associated with tamoxifen concentration ($r = -0.97$, $P < 0.0001$). The lowest tamoxifen concentration at which LDL propagation rate was significantly reduced was 5 μmol/L (Fig. 1, bottom).

DISCUSSION

In this study, we considered whether treatment with physiological levels of female hormones of the type commonly prescribed to postmenopausal women would influence susceptibility of LDL to in vitro lipid peroxidation. This hypothesis was tested in surgically postmenopausal cynomolgus macaques given CEE, MPA, or both hormones, tamoxifen, or no treatment during 12 weeks of being fed a cholesterol-containing diet to induce hypercholesterolemia similar to that in women (17, 18, 21–23). Female macaques share many features of reproductive physiology with human females, including similar circulating levels of sex steroids and the occurrence of a natural menopause (60, 61). We thus thought this animal model appropriate to consider whether supplementary female hormones would influence susceptibility of LDL to in vitro lipid peroxidation.

The principle findings of this study are that in surgically postmenopausal cynomolgus monkeys fed a controlled diet, neither CEE, MPA, both hormones, nor tamoxifen influenced susceptibility of LDL to in vitro lipid peroxidation as assessed by lag times and propagation rates. However, we found that LDL γ- and α-tocopherol, LDL molecular weight, and HDL cholesterol were independently associated with LDL lag times for in vitro lipid peroxidation. No previous study that investigated the effect of estrogen on susceptibility of LDL to in vitro lipid peroxidation investigated the contribution of any of these factors to effects of estrogen on susceptibility of LDL to in vitro lipid peroxidation. After accounting for the effects of the predictors of LDL lag time that we identified, LDL lag times for in vitro lipid peroxidation remained similar among all treatment groups. Propagation rates for LDL lipid peroxidation were not related to any of the plasma and LDL parameters that we investigated.

In agreement with our findings (Tables 4, 7), four studies in women treated with 0.625 mg CEE (18, 21, 22, 26), either alone (18, 22, 26) or with MPA (21, 26), also found that these treatments did not influence LDL lag time. Similarly, studies with oral 17β-estradiol valerate either alone or with MPA (22), and oral 17β-estradiol either alone

(23) or with cyclic norethisterone acetate (20), indicated that these estrogens did not affect LDL lag time. Limited data for estrogen monotherapy compared with combined treatment with estrogen/MPA (26), as well as studies in vitro with LDL supplemented with norgestrel, norethisterone, MPA and levonorgestrel (19, 62), suggested that progestins do not influence resistance of LDL to in vitro lipid peroxidation. Two other studies that lacked placebo controls reported that other oral estrogens increased LDL lag time (19, 24). In contrast to the results of most of the studies with oral estrogens, most (16, 17, 25, 26), but not all (22), studies of transdermal delivery of 17β-estradiol found that such treatment increased LDL lag time.

Some of the studies that investigated the influence of estrogen on susceptibility of LDL to in vitro lipid peroxidation reported data for propagation rates (18, 20–23, 25, 26, 39). Most of these studies (18, 20–23, 25, 26), and all studies that were randomized and placebo controlled (18, 20, 23), found that estrogen treatment either alone (18, 22, 23, 26) or with various progestins (20, 21, 25, 26) had no effect on the propagation rate for in vitro LDL lipid peroxidation. Consistent with the majority of those observations, we found that neither CEE, nor MPA, nor both hormones influenced LDL propagation rate (Tables 4, 7). The only study reporting data for the influence of oral tamoxifen treatment on in vitro LDL lipid peroxidation found that tamoxifen treatment modestly increased LDL lag time and slightly reduced LDL propagation rate (18). In contrast, we found that oral tamoxifen altered neither LDL lag time nor LDL propagation rate (Tables 4, 7), but supplementing LDL in vitro with tamoxifen at concentrations higher than those found therapeutically in women (63, 64) increased LDL lag time and reduced LDL propagation rate (Fig. 1, bottom).

One could speculate that differences in the form of estrogen might explain in part the differences between the results for LDL lag times for the studies of oral and transdermal estrogen replacement. However, one particularly well-controlled study of oral treatment with micronized 17β-estradiol found that this treatment had no influence on LDL lag time (23). Thus, it is likely that differences other than the form of estrogen account for the differences in results among studies. Plasma concentrations of estradiol were higher for the positive studies of transdermal 17β-estradiol, with mean values of 430–477 pmol/L (16, 17, 25, 26), compared with only 182 pmol/L for the negative study of transdermal 17β-estradiol (22) and 140–300 pmol/L for the negative studies with oral estrogens (18, 20, 22, 23, 26). Plasma concentrations of estradiol in all of these studies, as well as those of our monkeys supplemented with CEE (Table 2), were in the physiological range for premenopausal women (65, 66).

Limited data suggest that transdermal (27), but not oral (21) estrogens increase LDL vitamin E. Consistent with those data, we found that oral CEE (with or without MPA) influenced neither LDL α- nor γ-tocopherol (Table 3). Our value of LDL α-tocopherol is almost identical to that reported by Wander et al. (21), the only study of the effect of estrogen on oxidizability of LDL in postmenopausal women that investigated LDL vitamin E. In the United States, 54% of women consume less than the recommended value of 8 mg α-tocopherol per day (41). Another study showed that supplementation with vitamin E increased LDL lag time only for vitamin E-deficient subjects (32). One study showed that transdermal estrogen replacement that achieved plasma estradiol concentrations of 470 pmol/L did not further prolong LDL lag time for postmenopausal women supplemented with 800 IU of vitamin E (17). Neither that study nor any of the other studies in women that reported transdermal estrogen to increase LDL lag time (16, 25, 26) presented data for LDL vitamin E or vitamin E status. Thus, increased LDL vitamin E by transdermal estrogen in women with marginal vitamin E status may contribute to the greater frequency at which studies of transdermal estrogen reported increases in LDL lag time compared with studies of oral estrogen.

In this study, we found that LDL α-tocopherol was inversely related to LDL lag time after exposure to Cu^{2+} (Table 5) in a univariate analysis and this inverse association remained significant in a multivariate analysis (Table 6). In contrast, LDL lag time after AAPH oxidation was not related to LDL α-tocopherol. One study that investigated the influence of estrogens on susceptibility of LDL to in vitro oxidation reported values for LDL vitamin E (21). However, neither that study, nor any of the other studies that studied the influence of in vivo treatment with estrogen on susceptibility of LDL to in vitro lipid peroxidation, considered how LDL vitamin E might influence LDL lag time. In comparison to our results, several studies that did not involve estrogen found that LDL α-tocopherol was positively correlated with LDL lag time after Cu^{2+} oxidation (31, 32) while other studies found that LDL α-tocopherol was not related to lag time for Cu^{2+} mediated oxidation (67, 68). Other evidence suggests that α-tocopherol may be a prooxidant under some conditions (33, 34), consistent with our results for Cu^{2+}-mediated oxidation.

In contrast to LDL α-tocopherol, we found that LDL γ-tocopherol was positively associated with LDL lag time for Cu^{2+} oxidation, where it was the single most important determinant of LDL lag time (among the factors that we considered) in both univariate (Table 5) and multivariate analysis (Table 6). For AAPH oxidation, LDL γ-tocopherol was the second most important determinant of LDL lag time. As far as we are aware, this is the first report of a significant independent association of LDL γ-tocopherol with LDL lag time. However, one study reported that the sum of α- and γ-tocopherol was inversely related to LDL lag time mediated by AAPH (69). Also, another study reported that in phospholipid membranes, γ-tocopherol inhibited lipid hydroperoxide formation relatively better than α-tocopherol (70). Our observation regarding the positive association of γ-tocopherol with LDL lag time may be particularly important because γ-tocopherol is more abundant than α-tocopherol in several food oils, the major sources of vitamin E in the diet (71), while most studies of vitamin E supplementation have used α-tocopherol. Also, the single study of the effect of estrogen on LDL oxidizability in women reporting data for LDL vitamin E (21) reported

values of LDL γ-tocopherol about 5-fold those that we observed (Table 3). This suggests that the contribution of LDL γ-tocopherol to LDL oxidizability in women may be greater than that predicted for our monkeys (Table 6).

We considered whether HDL cholesterol might help explain variance in LDL lag time. In a univariate analysis we found the identical strength of association with LDL lag time after AAPH-mediated oxidation for both HDL cholesterol and LDL molecular weight, although the direction of the associations was inverse (Table 5). In a multivariate analysis, both HDL cholesterol and LDL molecular weight contributed significantly to LDL lag time, and the direction of the associations was unchanged (Table 6). These reciprocal relationships of LDL molecular weight and HDL cholesterol with LDL lag time are consistent with our observation that LDL molecular weight and HDL cholesterol were inversely correlated ($r = -0.37$, $P < 0.025$). In contrast, previous studies in human individuals reported that HDL cholesterol was positively correlated with LDL molecular size (54, 55).

In this study, we found that LDL molecular weight bore no relationship to lag time after Cu^{2+}-mediated oxidation. In contrast, we found that LDL molecular weight was inversely associated with LDL lag time for AAPH-mediated oxidation in a univariate analysis (Table 5) and that this association persisted in a multivariate analysis (Table 6). Only two studies of the influence of estrogen on resistance of LDL to in vitro lipid peroxidation assessed LDL molecular size (23, 39). One of those studies reported changes in LDL molecular weight that were not correlated with the hormone treatment, suggesting that such changes were due to other factors (39). The other study, which made the atypical observation that estrogen treatment did not influence LDL molecular size, found that estrogen treatment did not affect LDL lag time (23). In comparison to our results, a number of studies which did not involve estrogen found that lag times after Cu^{2+} oxidation were shorter for small and/or dense LDL compared with larger more buoyant LDL from the same individuals (31, 35–38). However, antioxidant content of small dense LDL was reduced compared with larger more buoyant LDL (31, 35–38); one study showed that the increased susceptibility of dense LDL to oxidation could be explained by the reduced antioxidant content (31). Apparently reduced LDL molecular size induced by estrogen, which is associated with increases in HDL cholesterol (29, 30), affects susceptibility of LDL to in vitro lipid peroxidation differently than reduced LDL molecular size in association with reduced HDL cholesterol (54, 55).

If estrogens have direct antioxidant activity, one might expect plasma concentrations of estradiol to be positively correlated with LDL lag time. In this study, we found that for all the monkeys considered as a group, plasma estradiol was not correlated with LDL lag time for either Cu^{2+} or AAPH oxidation (Table 5). However, when we limited our analysis to only those monkeys given CEE, we found that lag times for Cu^{2+} and AAPH oxidation were inversely correlated with plasma estradiol (Table 5). In comparison, two studies that reported that transdermal estradiol increased LDL lag time, one in women (16) and one in swine (72), reported that there was no relationship between plasma estradiol concentrations in supplemented individuals and LDL lag time.

In summary, using Cu^{2+} and AAPH, which initiate lipid peroxidation by different mechanisms (73, 74), we found that neither CEE, MPA, both hormones, nor tamoxifen influenced susceptibility of LDL to in vitro lipid peroxidation as indicated by lag times or propagation rates. In this study we confirmed the importance of LDL α-tocopherol in determining lag time after Cu^{2+}-mediated oxidation. This study identified an unexpected relationship between LDL molecular weight and lag time after AAPH-mediated oxidation which may suggest that implications of LDL molecular size on susceptibility of LDL to in vitro oxidation may depend to some degree on the metabolic process by which LDL molecular size is altered. In this study we also provide new evidence that LDL γ-tocopherol is an important determinant of susceptibility of LDL to both Cu^{2+}- and AAPH-mediated oxidation. This study also suggests that although LDL molecular size and HDL cholesterol have been correlated in some studies, these factors have independent effects on susceptibility of LDL to AAPH-mediated oxidation. Finally, although data might suggest a possible antioxidant effect of estrogens within the arterial wall (15, 53, 75–77), it seems likely that physiological concentrations of estrogen are unlikely to inhibit oxidation of LDL in the plasma compartment.

This study was supported by grant number HLP0145666 (M. R. A.) and by the Wake Forest University School of Medicine. The authors gratefully acknowledge the assistance of Deborah Golden with the monkeys. We are also grateful to Michael R. Marino and John S. Mason for their help with isolation and oxidation of LDL. D. C. S. is an Established Investigator of the American Heart Association. J. D. W. is a recipient of a National Center for Research Resources Career Development Award (RR000072).

Manuscript received 25 September 1998.

REFERENCES

1. Kuhn, F. E., and C. E. Rackley. 1993. Coronary artery disease in women—risk factors, evaluation, treatment, and prevention. *Arch. Intern. Med.* **153:** 2626–2636.
2. Chae, C. U., P. M. Ridker, and J. E. Manson. 1997. Postmenopausal hormone replacement therapy and cardiovascular disease. *Thromb. Haemost.* **78:** 770–780.
3. Barrett-Connor, E. 1997. Sex differences in coronary heart disease: why are women so superior? The 1995 Ancel Keys lecture. *Circulation.* **95:** 252–264.
4. Stampfer, M. J., and G. A. Colditz. 1991. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiological evidence. *Prev. Med.* **20:** 47–63.
5. Barrett-Connor, E., and T. L. Bush. 1991. Estrogen and coronary heart disease in women. *J. Am. Med. Assoc.* **265:** 1861–1867.
6. Guetta, V., A. A. Quyyumi, A. Prasad, J. A. Panza, M. Waclawiw, and R. O. Cannon. 1997. The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. *Circulation.* **96:** 2795–2801.
7. Schwenke, D. C. 1998. Antioxidants and atherogenesis. *J. Nutr. Biochem.* **9:** 424–445.
8. Haarbo, J., P. Leth-Espensen, S. Stender, and C. Christiansen.

1991. Estrogen monotherapy and combined estrogen-progestogen replacement therapy attenuate aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits. *J. Clin. Invest.* **87:** 1274–1279.
9. Haarbo, J., O. L. Svendsen, and C. Christiansen. 1992. Progestogens do not affect aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits. *Circ. Res.* **70:** 1198–1202.
10. Haarbo, J., and C. Christiansen. 1996. The impact of female sex hormones on secondary prevention of atherosclerosis in ovariectomized cholesterol-fed rabbits. *Atherosclerosis.* **123:** 139–144.
11. Adams, M. R., T. C. Register, D. L. Golden, J. D. Wagner, and J. K. Williams. 1997. Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **17:** 217–221.
12. Espeland, M. A., W. Applegate, C. D. Furberg, D. Lefkowitz, L. Rice, and D. Hunninghake. 1995. Estrogen replacement therapy and progression of intimal-medial thickness in the carotid arteries of postmenopausal women. *Am. J. Epidemiol.* **142:** 1011–1019.
13. Paech, K., P. Webb, G. G. J. M. Kuiper, S. Nilsson, J. A. Gustafsson, P. J. Kushner, and T. S. Scanlan. 1997. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science.* **277:** 1508–1510.
14. Nayfield, S. G., J. E. Karp, L. G. Ford, F. A. Dorr, and B. S. Kramer. 1991. Potential role of tamoxifen in prevention of breast cancer. *J. Natl. Cancer Inst.* **83:** 1450–1459.
15. Carew, T. E., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
16. Sack, M. N., D. J. Rader, and R. O. Cannon. 1994. Oestrogen and inhibition of oxidation of low-density lipoproteins in postmenopausal women. *Lancet.* **343:** 269–270.
17. Guetta, V., J. A. Panza, M. A. Waclawiw, and R. O. Cannon. 1995. Effect of combined 17 beta-estradiol and vitamin E on low-density lipoprotein oxidation in postmenopausal women. *Am. J. Cardiol.* **75:** 1274–1276.
18. Guetta, V., R. M. Lush, W. D. Figg, M. A. Waclawiw, and R. O. Cannon. 1995. Effects of the antiestrogen tamoxifen on low density lipoprotein concentrations and oxidation in postmenopausal women. *Am. J. Cardiol.* **76:** 1072–1073.
19. Schroder, J., M. Doren, B. Schneider, and M. Oettel. 1996. Are the antioxidative effects of 17 beta-estradiol modified by concomitant administration of a progestin? *Maturitas.* **25:** 133–139.
20. Nenseter, M. S., V. Volden, T. Berg, C. A. Drevon, L. Ose, and S. Tonstad. 1996. Effect of hormone replacement therapy on the susceptibility of low-density lipoprotein to oxidation among postmenopausal hypercholesterolaemic women. *Eur. J. Clin. Invest.* **26:** 1062–1068.
21. Wander, R. C., S. H. Du, S. O. Ketchum, and K. E. Rowe. 1996. Effects of interaction of RRR-alpha-tocopheryl acetate and fish oil on low-density-lipoprotein oxidation in postmenopausal women with and without hormone-replacement therapy. *Am. J. Clin. Nutr.* **63:** 184–193.
22. McManus, J., J. McEneny, W. Thompson, and I. S. Young. 1997. The effect of hormone replacement therapy on the oxidation of low density lipoprotein in postmenopausal women. *Atherosclerosis.* **135:** 73–81.
23. Brussaard, H. E., J. A. G. Leuven, C. Kluft, H. M. J. Krans, W. Vanduyvenvoorde, R. Buytenhek, A. Vanderlaarse, and H. M. G. Princen. 1997. Effect of 17 beta-estradiol on plasma lipids and LDL oxidation in postmenopausal women with type II diabetes mellitus. *Arterioscler. Thromb. Vasc. Biol.* **17:** 324–330.
24. Wilcox, J. G., H. Hwang, H. N. Hodis, A. Sevanian, F. Z. Stanczyk, and R. A. Lobo. 1997. Cardioprotective effects of individual conjugated equine estrogens through their possible modulation of insulin resistance and oxidation of low-density lipoprotein. *Fertil. Steril.* **67:** 57–62.
25. Koh, K. K., M. N. Bui, R. Mincemoyer, and R. O. Cannon. 1997. Effects of hormone therapy on inflammatory cell adhesion molecules in postmenopausal healthy women. *Am. J. Cardiol.* **80:** 1505–1507.
26. Koh, K. K., R. Mincemoyer, M. N. Bui, G. Csako, F. Pucino, V. Guetta, M. Waclawiw, and R. O. Cannon. 1997. Effects of hormone-replacement therapy on fibrinolysis in postmenopausal women. *N. Engl. J. Med.* **336:** 683–690.
27. Clemente, C., M. G. Caruso, P. Berloco, A. Buonsante, B. Giannandrea, and A. DiLeo. 1996. Alpha-tocopherol and beta-carotene serum levels in post-menopausal women treated with transdermal estradiol and oral medroxyprogesterone acetate. *Horm. Metab. Res.* **28:** 558–561.
28. Wagner, J. D., D. C. Schwenke, L. Zhang, D. Apple-Baumbowden, J. D. Bagdade, and M. R. Adams. 1997. Effects of short-term hormone replacement therapies on low-density lipoprotein metabolism in cynomolgus monkeys. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1128–1134.
29. Campos, H., F. M. Sacks, B. W. Walsh, I. Schiff, M. A. Ohanesian, and R. M. Krauss. 1993. Differential effects of estrogen on low-density lipoprotein subclasses in healthy postmenopausal women. *Metabolism.* **42:** 1153–1158.
30. Griffin, B., E. Farish, D. Walsh, J. Barnes, M. Caslake, J. Shepherd, and D. Hart. 1993. Response of plasma low density lipoprotein subfractions to oestrogen replacement therapy following surgical menopause. *Clin. Endocrinol.* **39:** 463–468.
31. Tribble, D. L., J. J. M. Vandenberg, P. A. Motchnik, B. N. Ames, D. M. Lewis, A. Chait, and R. M. Krauss. 1994. Oxidative susceptibility of low density lipoprotein subfractions is related to their ubiquinol-10 and alpha-tocopherol content. *Proc. Natl. Acad. Sci. USA.* **91:** 1183–1187.
32. Winklhofer-Roob, B. M., O. Ziouzenkova, H. Puhl, H. Ellemunter, P. Greiner, G. Muller, M. A. Vanthof, H. Esterbauer, and D. H. Shmerling. 1995. Impaired resistance to oxidation of low density lipoprotein in cystic fibrosis: improvement during vitamin E supplementation. *Free Radical Biol. Med.* **19:** 725–733.
33. Witting, P. K., V. W. Bowry, and R. Stocker. 1995. Inverse deuterium kinetic isotope effect for peroxidation in human low-density lipoprotein (LDL): a simple test for tocopherol-mediated peroxidation of LDL lipids. *FEBS Lett.* **375:** 45–49.
34. Ingold, K. U., V. W. Bowry, R. Stocker, and C. Walling. 1993. Autoxidation of lipids and antioxidation by alpha-tocopherol and ubiquinol in homogeneous solution and in aqueous dispersions of lipids—unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **90:** 45–49.
35. de Graaf, J., H. L. Hak-Lemmers, M. P. Hectors, P. N. Demacker, J. C. Hendriks, and A. F. Stalenhoef. 1991. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler. Thromb.* **11:** 298–306.
36. Reaven, P. D., B. J. Grasse, and D. L. Tribble. 1994. Effects of linoleate-enriched and oleate-enriched diets in combination with alpha-tocopherol on the susceptibility of LDL and LDL subfractions to oxidative modification in humans. *Arterioscler. Thromb.* **14:** 557–566.
37. Tribble, D. L., P. M. Thiel, J. J. M. Vandenberg, and R. M. Krauss. 1995. Differing alpha-tocopherol oxidative lability and ascorbic acid sparing effects in buoyant and dense LDL. *Arterioscler. Thromb. Vasc. Biol.* **15:** 2025–2031.
38. Reaven, P., B. Grasse, and J. Barnett. 1996. Effect of antioxidants alone and in combination with monounsaturated fatty acid-enriched diets on lipoprotein oxidation. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1465–1472.
39. Mckinney, K. A., P. B. Duell, D. L. Wheaton, D. L. Hess, P. E. Patton, H. G. Spies, and K. A. Burry. 1997. Differential effects of subcutaneous estrogen and progesterone on low-density lipoprotein size and susceptibility to oxidation in postmenopausal rhesus monkeys. *Fertil. Steril.* **68:** 525–530.
40. Williams, J. K., J. D. Wagner, Z. Li, D. L. Golden, and M. R. Adams. 1997. Tamoxifen inhibits arterial accumulation of LDL degradation products and progression of coronary artery atherosclerosis in monkeys. *Arterioscler. Thromb. Vasc. Biol.* **17:** 403–408.
41. Millen, B. E., P. A. Quatromoni, M. M. Franz, B. E. Epstein, L. A. Cupples, and D. L. Copenhafer. 1997. Population nutrient intake approaches dietary recommendations: 1991 to 1995 Framingham Nutrition Studies. *J. Am. Diet. Assoc.* **97:** 742–749.
42. Lipid Research Clinics Program. 1982. Manual of Laboratory Operations. Lipid and Lipoprotein Analysis. Department of Health, Education, and Welfare publication number (NIH) 75-629, Washington DC: US Government Printing Office.
43. Wagner, J. D., M. A. Martino, M. J. Jayo, M. S. Anthony, T. B. Clarkson, and W. T. Cefalu. 1996. The effects of hormone replacement therapy on carbohydrate metabolism and cardiovascular risk factors in surgically postmenopausal cynomolgus monkeys. *Metabolism.* **45:** 1254–1262.

44. Schwenke, D. C. 1997. Gender differences in intima-media permeability to low-density lipoprotein at atherosclerosis-prone aortic sites in rabbits: lack of effect of 17β-estradiol. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2150–2157.
45. Schwenke, D. C. 1997. Comparison of aorta and pulmonary artery. II. LDL transport and metabolism correlate with susceptibility to atherosclerosis. *Circ. Res.* **81:** 346–354.
46. Epler, K. S., R. G. Ziegler, and N. E. Craft. 1993. Liquid chromatographic method for the determination of carotenoids, retinoids and tocopherols in human serum and in food. *J. Chromatogr. Biomed. Appl.* **619:** 37–48.
47. Elinder, L. S., and G. Walldius. 1992. Simultaneous measurement of serum probucol and lipid-soluble antioxidants. *J. Lipid Res.* **33:** 131–137.
48. Schwenke, D. C., and S. R. Behr. 1998. Vitamin E combined with selenium inhibit atherosclerosis in hypercholesterolemic rabbits independently of effects on plasma cholesterol concentrations. *Circ. Res.* **83:** 366–377.
49. Parthasarathy, S., J. C. Khoo, E. Miller, J. Barnett, J. L. Witztum, and D. Steinberg. 1990. Low density lipoprotein rich in oleic acid is protected against oxidative modification: implications for dietary prevention of atherosclerosis. *Proc. Natl. Acad. Sci. USA.* **87:** 3894–3898.
50. Esterbauer, H., G. Striegl, H. Puhl, and M. Rotheneder. 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **6:** 67–75.
51. Ramos, P., S. P. Gieseg, B. Schuster, and H. Esterbauer. 1995. Effect of temperature and phase transition on oxidation resistance of low density lipoprotein. *J. Lipid Res.* **36:** 2113–2128.
52. Snedecor, G. W. and W. G. Cochran. 1979. Statistical Methods. The Iowa State University Press, 6th ed. Ames, IA. 3–593.
53. Wagner, J. D., L. Zhang, J. K. Williams, T. C. Register, D. M. Ackerman, B. Witta, T. B. Clarkson, and M. R. Adams. 1996. Esterified estrogens with and without methyltestosterone decrease arterial LDL metabolism in cynomolgus monkeys. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1473–1480.
54. Campos, H., J. J. Genest, E. Blijlevens, J. R. McNamara, J. L. Jenner, J. M. Ordovas, P. W. F. Wilson, and E. J. Schaefer. 1992. Low density lipoprotein particle size and coronary artery disease. *Arterioscler. Thromb.* **12:** 187–195.
55. Coresh, J., P. O. Kwiterovich, H. H. Smith, and P. S. Bachorik. 1993. Association of plasma triglyceride concentration and LDL particle diameter, density, and chemical composition with premature coronary artery disease in men and women. *J. Lipid Res.* **34:** 1687–1697.
56. Barakat, H. A., J. W. Carpenter, V. D. Mclendon, P. Khazanie, N. Leggett, J. Heath, and R. Marks. 1990. Influence of obesity, impaired glucose tolerance, and NIDDM on LDL structure and composition—possible link between hyperinsulinemia and atherosclerosis. *Diabetes.* **39:** 1527–1533.
57. Allen, D. M., and F. B. Cady. 1982. Analyzing experimental data by regression. Wadsworth, Inc., Belmont, CA. 138–141.
58. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
59. Yang, C-Y., S-H. Chen, S. H. Gianturco, W. A. Bradley, J. T. Sparrow, M. Tanimura, W-H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F-S. Lee, Z-W. Gu, A. M. Gotto, and L. Chan. 1986. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature.* **323:** 738–742.
60. Adams, M. R., J. R. Kaplan, D. R. Koritnik, and T. B. Clarkson. 1987. Pregnancy-associated inhibition of coronary artery atherosclerosis in monkeys. Evidence of a relationship with endogenous estrogen. *Arteriosclerosis.* **7:** 378–384.
61. Clarkson, T. B., C. L. Hughes, and K. P. Klein. 1995. The nonhuman primate model of the relationship between gonadal steroids and coronary heart disease. *Prog. Cardiovasc. Dis.* **38:** 189–198.
62. McManus, J., J. McEneny, I. S. Young, and W. Thompson. 1996. The effect of various oestrogens and progestogens on the susceptibility of low density lipoproteins to oxidation in vitro. *Maturitas.* **25:** 125–131.
63. Lien, E. A., E. Solheim, and P. M. Ueland. 1991. Distribution of tamoxifen and its metabolites in rat and human tissues during steady-state treatment. *Cancer Res.* **51:** 4837–4844.
64. Lien, E. A., E. Solheim, O. A. Lea, S. Lundgren, S. Kvinnsland, and P. M. Ueland. 1989. Distribution of 4-hydroxy-N-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res.* **49:** 2175–2183.
65. Schaefer, E. J., S. Lamon-Fava, D. Spiegelman, J. T. Dwyer, A. H. Lichtenstein, J. R. Mcnamara, B. R. Goldin, M. N. Woods, A. Morrill-LaBrode, E. Hertzmark, C. Longcope, and S. L. Gorbach. 1995. Changes in plasma lipoprotein concentrations and composition in response to a low-fat, high-fiber diet are associated with changes in serum estrogen concentrations in premenopausal women. *Metabolism.* **44:** 749–756.
66. Boyd, N. F., G. A. Lockwood, C. V. Greenberg, L. J. Martin, and D. L. Tritchler. 1997. Effects of a low-fat high-carbohydrate diet on plasma sex hormones in premenopausal women: results from a randomized controlled trial. *Br. J. Cancer.* **76:** 127–135.
67. Kleinveld, H. A., H. L. M. Haklemmers, A. F. H. Stalenhoef, and P. N. M. Demacker. 1992. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation—application of a short procedure for isolating low-density lipoprotein. *Clin. Chem.* **38:** 2066–2072.
68. Kontush, A., C. Hubner, B. Finckh, A. Kohlschutter, and U. Beisiegel. 1996. How different constituents of low density lipoprotein determine its oxidizability by copper: a correlational approach. *Free Radical Res.* **24:** 135–147.
69. Frei, B., and J. M. Gaziano. 1993. Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and ion-independent oxidation. *J. Lipid Res.* **34:** 2135–2145.
70. Christen, S., A. A. Woodall, M. K. Shigenaga, P. T. Southwell-Keely, M. W. Duncan, and B. N. Ames. 1997. Gamma-tocopherol traps mutagenic electrophiles such as NOx and complements alpha-tocopherol: physiological implications. *Proc. Natl. Acad. Sci. USA.* **94:** 3217–3222.
71. Papas, A. M. 1993. Oil-soluble antioxidants in foods. *Toxicol. Ind. Health.* **9:** 123–150.
72. Keaney, J. F., Jr., G. T. Shwaery, A. M. Xu, R. J. Nicolosi, J. Loscalzo, T. L. Foxall, and J. A. Vita. 1994. 17 Beta-estradiol preserves endothelial vasodilator function and limits low-density lipoprotein oxidation in hypercholesterolemic swine. *Circulation.* **89:** 2251–2259.
73. Niki, E. 1990. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol.* **186:** 100–108.
74. Thomas, J. P., B. Kalyanaraman, and A. W. Girotti. 1994. Involvement of preexisting lipid hydroperoxides in Cu^{2+}-stimulated oxidation of low-density lipoprotein. *Arch. Biochem. Biophys.* **315:** 244–254.
75. Wagner, J. D., M. R. Adams, D. C. Schwenke, and T. B. Clarkson. 1993. Oral contraceptive treatment decreases arterial low density lipoprotein degradation in female cynomolgus monkeys. *Circ. Res.* **72:** 1300–1307.
76. Wagner, J. D., R. W. St. Clair, D. C. Schwenke, C. A. Shively, M. R. Adams, and T. B. Clarkson. 1992. Regional differences in arterial low density lipoprotein metabolism in surgically postmenopausal cynomolgus monkeys. Effects of estrogen and progesterone replacement therapy. *Arterioscler. Thromb.* **12:** 717–726.
77. Maziere, C., M. Auclair, M-F. Ronveaux, S. Salmon, R. Santus, and J. C. Maziere. 1991. Estrogens inhibit copper and cell-mediated modification of low density lipoprotein. *Atherosclerosis.* **89:** 175–182.