Estrogen Replacement Decreases the Level of Antibodies Against Oxidized Low-Density Lipoprotein in Postmenopausal Women With Coronary Heart Disease

N. Hoogerbrugge, M.C. Zillikens, H. Jansen, K. Meeter, J.W. Deckers, and J.C. Birkenhäger

The effect of estrogen replacement therapy (ERT) on plasma lipid concentrations and oxidation parameters was studied in 25 hypercholesterolemic women with coronary heart disease (CHD). During ERT, the low-density lipoprotein cholesterol (LDLc) concentration decreased from 4.31 ± 0.72 to 3.85 ± 0.62 mmol/L ($P < .01$) and high-density lipoprotein cholesterol (HDLc) increased from 1.42 ± 0.30 to 1.55 ± 0.33 mmol/L ($P < .01$). The concentration of autoantibodies against oxidized LDL decreased from 25.9 ± 22.0 to 22.7 ± 19.9 mg/L ($P < .05$), indicating that ERT may have antioxidative effects in vivo. The lag time to oxidation and the LDL subclass pattern did not change. Analysis of the influence of smoking on the efficacy of ERT showed that ERT significantly affected LDLc and HDLc concentrations in 15 nonsmoking women. However, in 10 cigarette smokers, no significant changes in LDLc or HDLc levels were observed. Smoking did not affect the concentration of autoantibodies to oxidized LDL or the lag time. Medroxyprogesterone acetate ([MPA] 10 mg daily) added to ERT decreased HDLc by 9% ($P < .01$) but did not affect the LDLc level, LDL subclass pattern, or lag time. In conclusion, ERT may have antioxidative effects in vivo and favorably affects dyslipidemia in hypercholesterolemic women with CHD, especially when they refrain from smoking.

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ESTROGEN REPLACEMENT THERAPY (ERT) reduces the risk for cardiovascular disease in healthy postmenopausal women by approximately 50%.[1] This protective effect of ERT is only partly explained by antiatherogenic changes in the lipoprotein levels,[2,3] and may include inhibition of low-density lipoprotein (LDL) oxidation.[4-6]

Autoantibodies to epitopes of oxidized LDL, such as malondialdehyde (MDA), may be used to evaluate the impact of LDL oxidation in vivo.[7] It was shown that these autoantibodies react with human atherosclerotic plaques[8,9] and are associated with the progression of atherosclerosis.[10] Direct measurement of the oxidative processing of LDL is complicated, as it is likely this process is modified in vitro and the primary location may not be the bloodstream. An accepted approach is the measurement of the time course of peroxidation product formation in LDL following exposure to copper in vitro, ie, the lag time.[11]

The susceptibility of LDL for oxidation depends on LDL size: small dense LDL particles are more susceptible to oxidation than larger LDL particles.[12,13] Postmenopausal women have a preponderance of small dense LDL particles, whereas premenopausal women most frequently have large particles.[14] These data suggest that LDL size and perhaps LDL oxidation are related to estrogen levels.

In the present study, we assessed the effects of ERT with and without additional progestagen on plasma lipoprotein concentrations, LDL oxidation, and LDL particle size in smoking and nonsmoking hypercholesterolemic women with coronary heart disease (CHD).

Postmenopausal women with CHD are at high risk for new cardiovascular complications and are therefore excellent candidates for therapy to reduce cardiovascular risk like ERT. Among these women, there is a high prevalence of smokers. Smoking has been reported to exert an antiestrogenic effect in the treatment of osteoporosis[15,16] and may enhance the susceptibility of LDL to oxidative modification.[17] Whether cigarette smoking affects the favorable effects of ERT on plasma lipoprotein levels is not known. To eliminate the risk of endometrial carcinoma linked to unopposed estrogen, the addition of progestagen is recommended in women with a uterus. However, progestagen counteracts the favorable changes in high-density lipoprotein cholesterol (HDLc) induced by ERT. It is not known whether additional progestagen has antioxidant activity.

SUBJECTS AND METHODS

Patients

Twenty-five postmenopausal women aged 58 ± 5 years with coronary artery disease (prior myocardial infarction or angina pectoris during exercise test) and a plasma cholesterol level of 6.0 to 9.0 mmol/L were recruited. Women were characterized as postmenopausal if they had not had menses for at least 1 year. Women who used estrogen or lipid-lowering medication in the 3 months preceding the study were excluded. Other exclusion criteria were a fasting triglyceride (TG) concentration higher than 2.5 mmol/L, apolipoprotein (apo) E2/E2 phenotype, myocardial infarction within the prior 6 months, diabetes mellitus, hypothyroidism, or other causes of secondary hyperlipidemia (abnormal renal or hepatic function), and gross obesity (body mass index $\geq$35 kg/m^2). Written informed consent was obtained from all participants. The study was approved by the Medical Ethics Committee of the University Hospital Dijkzigt.

Protocol

In the first part of the study, the effects of ERT on plasma lipids were studied in a double-blind, randomized, placebo-controlled crossover trial. All of the women were given placebo medication for 14 days to identify those unlikely to adhere to the protocol. After this period, the women were randomized to one of two treatment arms: 8 weeks of estradiol valerate 2 mg/d followed by 8 weeks of placebo, or 8 weeks of placebo followed by estradiol valerate for 8 weeks. This part of the

From the Departments of Internal Medicine III and Cardiology, University Hospital Dijkzigt Rotterdam, Rotterdam, The Netherlands.

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Address reprint requests to N. Hoogerbrugge, MD, PhD, University Hospital Dijkzigt, Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

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study was double-blind. In the second part of the study, the effects of medroxyprogesterone acetate (MPA) were studied. All women were treated (open) with estradiol valerate 2 mg/d for 8 weeks followed by additional use of MPA 10 mg/d for 2 weeks.

The estrogen dose chosen was optimal for prevention of postmenopausal bone loss, and is equipotent to 0.625 mg conjugated estradiol. Medroxyprogestagen was chosen because of its minor effects on plasma lipids as compared with other progestagens and the established endometrium protection. Medication was taken orally. Study medications and placebo were prepared in identical forms. The women were instructed not to change their eating habits during the trial. Diet was evaluated at the beginning and end of the study by a dietician and did not change.

Study Compliance

All 25 women entering the study completed the first phase of the crossover trial, and 23 women completed the second part of the study. One patient withdrew after the first phase because of mental depression. One woman had to be excluded because a hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitor was inadvertently used. Compliance to the medication was checked by tablet count. Every patient used greater than 90% of the provided number of tablets per period. The concentration of serum estradiol and estrone was measured in the open treatment phase.

Analytical Methods

Fasting blood samples were drawn into vacutainer tubes containing 1.5 g/L Na_2EDTA with the subject seated. Plasma was prepared from the blood within 2 hours. Sucrose was added to prevent lipoprotein modification, with a final sucrose concentration of 0.6%.[18] The plasma was stored until analysis at −80°C. All samples from one subject were analyzed simultaneously.

Cholesterol and TG were determined enzymatically. The plasma HDLc level was measured after precipitation of very–low-density lipoprotein (VLDL) and LDL by addition of manganese chloride. The supernatant was further fractionated into HDL_2 and HDL_3 by precipitation of HDL_2 with 0.14% dextran sulfate.[19] Apo A-1 and apo B levels were estimated by an immunoturbidimetric method using commercially available kits from Dako (Glostrup, Denmark). Lipoprotein lipase (LPL) and hepatic lipase (HL) activity was measured using an immunochemical method[20] in plasma collected 20 minutes after contralateral intravenous administration of heparin 50 U/kg (Leo Pharmaceutical Products, Weesp, The Netherlands). Apo E isoforms were determined by isoelectric focusing.[21] Lipoproteins were isolated within 24 hours by density gradient ultracentrifugation for 24 hours at 200,000 × *g* at 4°C using a SW-41 rotor in a Beckman (Irvine, CA) L5-50 ultracentrifuge.[22] Serum estradiol and estrone levels were determined using immunoassay kits from Diagnostic Products (Los Angeles, CA) and Diagnostics Systems Laboratories (Webster, TX), respectively.

LDL Oxidation

To prevent oxidation of lipoproteins during ultracentrifugation, 0.1 mmol/L EDTA and 0.005% thimerosal were added to the gradient solutions. Before ultracentrifugation, the solutions were gassed by nitrogen to remove the oxygen. Immediately after isolation, part (300 μL) of the LDL fraction was desalted by filtration over PD-10 columns (Sephadex G-25M; Pharmacia, Uppsala, Sweden). LDL samples were diluted to a final protein concentration of 50 ng/L in 0.01 mmol/L phosphate-buffered saline. The oxidizability of LDL was assessed with a spectrophotometric technique essentially as described by Esterbauer et al.[11] Oxidation of LDL was started by addition of 10 μL 166-μmol/L $CuCl_2$ to 1 mL diluted LDL. Diene formation was evaluated by monitoring the change in absorbance at 234 nm every 2 minutes for 16 hours at 21°C. The lag time was defined as the interval between initiation of oxidation (time zero) and the intercept of the tangent of the slope of the absorbance curve during the propagation phase. The lag time represents the phase of LDL resistance to oxidation before the rapid conversion of polyunsaturated fatty acids of LDL to conjugated hydroperoxides.

LDL Subclass Pattern Determination

Gradient gel electrophoresis using self-made 2% to 16% nondenaturing polyacrylamide gels was performed to separate LDL subfractions.[20] Samples were subjected to electrophoresis for approximately 24 hours, for a total of 3,000 volt hours. They were stained with Oil Red O. The location of individual bands was determined by comparison to known control bands of a pooled plasma standard in the scanning of each gel. Predominantly large LDL is called pattern A, and predominantly small LDL pattern B.[20]

Autoantibodies Against MDA-Modified LDL

MDA-modified LDL (MDA-LDL) was prepared as described by Palinski et al.[23] The concentration of autoantibodies against MDA-LDL was determined by enzyme-linked immunosorbent assay exactly as described previously by our group.[24] Under standard conditions, the within-assay coefficient of variation in each series of determinations was 2.8%.

Statistical Analysis

All data are presented as the mean ± SD. ERT effects were tested by a one-sample *t* test on all within-subject differences between the two treatments. The carryover effect was evaluated, but did not exist. Differences between groups were evaluated for significance using the Student *t* test or by ANOVA corrected for multiple comparisons. Simple correlations between variables were calculated using the Pearson correlation test. The level of significance was set at $P < .05$.

RESULTS

Effects of ERT on Plasma Lipid Concentrations and Lipase Activities

The baseline characteristics of age, years after menopause, diet composition, alcohol intake, family history, and body mass index and the plasma lipid parameters of the groups starting with ERT or placebo were comparable. There were five smokers in each group. The use of concomitant medication was comparable, eg, eight and nine patients used β-blocking agents and one and three patients used diuretics. The mean body mass index was 25.1 ± 3.2 kg/m^2, and the duration of the postmenopausal period was 12.4 ± 4.7 years. Baseline laboratory data and ERT effects compared with placebo effects on plasma lipid parameters are listed in Table 1. Treatment with 2 mg estradiol valerate for 8 weeks resulted in an 11% decrease in LDLc ($P < .01$). There was a linear relation between the estradiol level on ERT and the ERT-induced change in LDLc concentration ($r = .57$, $P < .01$).

On ERT, HDLc and apo A-1 both increased by 10% ($P < .01$) and TG by 22% ($P < .01$; Table 1). This increase in total plasma TG was due to an increase of TG in the IDL, LDL, and HDL fraction. TG levels in these fractions on placebo and on ERT were 0.13 ± 0.03 and 0.16 ± 0.06 mmol/L ($P < .05$), 0.29 ± 0.07 and 0.35 ± 0.08 mmol/L ($P < .001$), and 0.24 ± 0.04 and 0.31 ± 0.11 mmol/L ($P < .001$) for IDL, LDL, and HDL, respectively. The TG concentration within the VLDL fraction was 1.15 ± 0.71 mmol/L on placebo and 1.33 ± 0.73

Table 1. Effects of 2 mg Estradiol Valerate Versus Placebo for 8 Weeks in 25 Postmenopausal Women With CHD

Parameter	Baseline	Placebo	Estradiol	Difference
Cholesterol (mmol/L)	6.81 ± 1.04	6.82 ± 1.05	6.61 ± 0.78	−0.20 ± 0.15
TG (mmol/L)	1.80 ± 0.69	1.80 ± 0.81	2.22 ± 0.95	0.41 ± 0.13†
HDLc (mmol/L)	1.37 ± 0.25	1.42 ± 0.30	1.55 ± 0.33	0.14 ± 0.05†
HDL_2 (mmol/L)	0.19 ± 0.15	0.20 ± 0.14	0.27 ± 0.19	0.07 ± 0.04
HDL_3 (mmol/L)	1.18 ± 0.18	1.22 ± 0.21	1.29 ± 0.23	0.07 ± 0.03*
VLDLc (mmol/L)	0.78 ± 0.40	0.67 ± 0.44	0.76 ± 0.41	0.09 ± 0.06
IDLc (mmol/L)	0.39 ± 0.15	0.36 ± 0.15	0.41 ± 0.16	0.05 ± 0.03
LDLc (mmol/L)	4.15 ± 0.89	4.31 ± 0.72	3.85 ± 0.62	−0.45 ± 0.15†
Apo A-1 (μg/L)	141 ± 15	143 ± 14	158 ± 20	16 ± 3†
Apo B (μg/L)	147 ± 23	147 ± 21	146 ± 23	1 ± 3

*$P < .05$, †$P < .01$: placebo *v* estradiol.

mmol/L on ERT (nonsignificant), respectively. ERT resulted in a 33% decrease in HL activity ($P < .001$). There was a linear relation between the estradiol level on ERT and the ERT-induced change in HL activity ($r = .50$, $P = .01$; Fig 1). The activity of LPL was not affected by ERT (Table 2).

Effects of ERT on LDL Size and LDL Oxidation Parameters

ERT decreased the level of autoantibodies against oxidized LDL by 12% ($P < .05$; Table 2). Small LDL particles, characterized as LDL pattern B, were present in 16 of 25 patients at baseline. After 8 weeks of ERT, the LDL pattern changed slightly but not significantly toward smaller particles: two of three patients with LDL pattern A had a shift to A/B, and two of five patients with LDL pattern A/B had a change to pattern B. Only one patient had a change to a larger LDL particle. This patient had pattern B during placebo and A/B on ERT. Changes in HL activity were not related to changes in LDL pattern. The lag time, representing LDL oxidation in vitro, was not affected by ERT (Table 2).

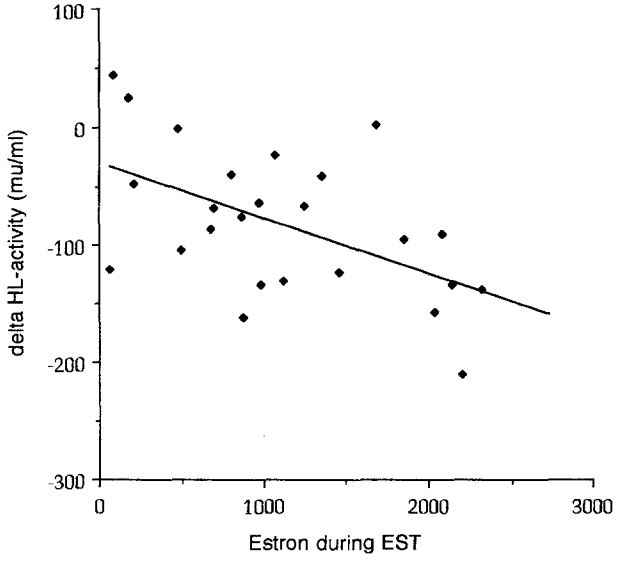

Fig 1. Relation between the estradiol concentration during ERT and the difference in HL activity before and during ERT ($r = .50$, $P = .01$).

Table 2. Effects of 2 mg Estradiol Valerate for 8 Weeks on LDL Subclass Pattern and Parameters of LDL Oxidizability in 25 Hypercholesterolemic Postmenopausal Women With CHD

Parameter	Placebo	Estradiol
LDL subclass (n)		
Pattern A	3	1
Pattern A/B	5	6
Pattern B	17	18
HL (U/L)	324 ± 103	250 ± 71†
LPL (U/L)	165 ± 46	173 ± 49
Lag time (min)	126 ± 20	127 ± 20
Autoantibodies to MDA-LDL (mg/L)	25.9 ± 22.0	22.7 ± 19.9*

*$P < .05$, †$P < .01$: placebo *v* estradiol.

Effects of MPA in Postmenopausal Women Using ERT

The additional use of 10 mg MPA for 2 weeks in 23 women with CHD using ERT caused a 10% decrease in HDLc, due to a decrease in both HDL_2 and HDL_3, and in apo A-1 ($P < .001$). LDLc and TG concentrations and HL activity were not affected by this additional treatment with MPA (Table 3). LPL activity decreased 10%, from 174 ± 36 to 156 ± 43 U/L ($P < .05$). The LDL lag time to copper-induced oxidation and the LDL pattern were not changed by additional MPA treatment (data not shown).

Effects of Smoking on the Efficacy of ERT

Smokers and nonsmokers were comparable with respect to age, years after menopause, diet composition, alcohol intake, family history, and physical characteristics, including body mass index (data not shown). Smokers used a mean of 12 ± 9 cigarettes per day (range, 2 to 30). HDLc concentrations at baseline were significantly lower in smokers compared with nonsmokers, 1.26 ± 0.23 and 1.45 ± 0.23 mmol/L, respectively ($P < .05$). The lower HDLc concentration in smokers was due to a significantly lower HDL_3 concentration, 1.06 ± 0.12 versus 1.27 ± 0.17 mmol/L ($P < .01$). Apo A-1 was also lower in smokers compared with nonsmokers, 133 ± 14 versus 146 ± 13 μg/L ($P = .01$). At baseline, no differences were observed between smokers and nonsmokers for cholesterol, TG, LDLc, and apo B concentrations (data not shown). The effects of 2 mg estradiol valerate for 8 weeks on plasma lipids in smokers and nonsmokers are shown in Table 4. In nonsmoking patients, ERT

Table 3. Additional Effects of 10 mg MPA for 14 Days in 23 Postmenopausal Women on ERT (n = 23)

Parameter	ERT	ERT + MPA
Cholesterol (mmol/L)	6.53 ± 0.84	6.35 ± 0.85*
TG (mmol/L)	2.01 ± 0.74	1.91 ± 0.69
HDLc (mmol/L)	1.58 ± 0.30	1.42 ± 0.26†
HDL_2 (mmol/L)	0.22 ± 0.14	0.15 ± 0.11†
HDL_3 (mmol/L)	1.36 ± 0.25	1.26 ± 0.21†
LDLc (mmol/L)	3.84 ± 0.73	3.85 ± 0.71
Apo A-1 (μg/L)	162 ± 19	148 ± 19†
Apo B (μg/L)	138 ± 22	139 ± 21
LPL (U/L)	174 ± 36	156 ± 43*
HL (U/L)	245 ± 90	260 ± 93
Lag time (min)	125 ± 20	125 ± 23

Abbreviation: ERT, estrogen replacement therapy.
*$P < .05$, †$P < .01$: ERT *v* ERT + MPA.

Table 4. Effects of 8 Weeks of 2 mg Estradiol Valerate Versus Placebo in Nonsmoking (n = 15) and Smoking (n = 10) Hypercholesterolemic Postmenopausal Women With CHD

Group	Cholesterol (mmol/L)	TG (mmol/L)	LDLc (mmol/L)	HDLc (mmol/L)	Apo A-1 (μg/L)
Nonsmokers					
Value	−0.35 ± 0.23	0.47 ± 0.19*	−0.51 ± 0.23*	0.18 ± 0.06*	21 ± 3*
Change (%)	−4	24	−13	13	13
Smokers					
Value	−0.03 ± 0.15	0.33 ± 0.18	−0.40 ± 0.19	0.07 ± 0.06	8 ± 5
Change (%)	0	20	−9	5	5

*$P < .05$ *v* placebo.

significantly affected LDLc, HDLc, apo A-1, and TG. There were no significant effects of ERT on plasma lipids in smokers (Table 4). Smoking did not affect the MPA-induced decrease in HDLc and apo A-1.

In the second study period, all women were treated with ERT. During this treatment, estradiol and estrone concentrations were measured. Significantly lower estrone and estradiol levels were seen in smokers compared with nonsmokers ($P < .01$; Fig 2). The concentration of sex hormone–binding globulin was comparable in smokers and nonsmokers both at baseline and during ERT.

No differences were observed in the LDL pattern, concentration of autoantibodies to LDL, or LDL oxidation lag time between smokers and nonsmokers (data not shown). In nonsmokers, ERT decreased the effect on autoantibodies to oxidized LDL by 17% ($P < .04$). In smokers, no significant effect of ERT on the concentration of autoantibodies was observed. ERT did not affect LPL activity, the lag time, or the LDL pattern in either smokers or nonsmokers (data not shown).

DISCUSSION

In postmenopausal women with CHD, ERT decreased the concentration of autoantibodies against oxidized LDL. This reduction may indicate that a physiological dose of estrogen decreases LDL oxidation in vivo. However, a decrease in the autoantibody concentration can result either from a decrease in antibody production due to a decrease in LDL oxidation or from an effective elimination of antigen-antibody complexes. The latter mechanism may not be antiatherogenic. Results from studies that address the association of antibodies to LDL and atherosclerosis are conflicting. A relation with the autoantibody level and the progression of atherosclerosis has been reported by some investigators,[10,25-27] but was not observed by others.[28,29] In our study, ERT did not affect the susceptibility of LDL particles to oxidation in vitro as measured by the lag time.

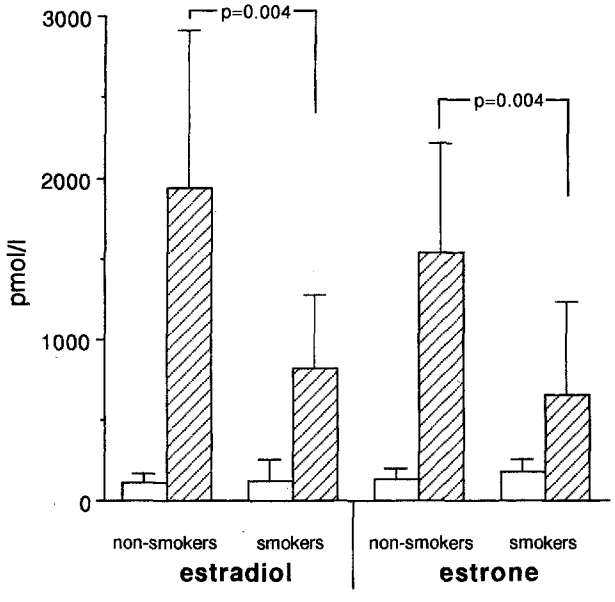

Fig 2. Effect of smoking on plasma concentrations of estrone and estradiol before (□) and after (*) substitution treatment with 2 mg estradiol valerate.

Previously, it has been shown that estrogen selectively reduces the concentration of the larger and lighter LDL particles in normocholesterolemic healthy women.[30] The lack of a significant effect of ERT on the LDL pattern in our population may result from the fact that the majority of the patients had LDL pattern B. The effect of ERT on LDL size can be direct, but can also be mediated by the estrogen-induced increase in the TG concentration and the decrease in HL activity. It has been shown that LDL size has a negative correlation with total TG levels and HL activity,[31] whereas the effect of TG on the LDL pattern is much stronger than the effect of HL activity.[20] In our study, the ERT-induced increase in the TG concentration was related to changes toward a smaller LDL particle size. On the other hand, the ERT-induced decrease in HL activity did not relate to changes in the LDL pattern.

ERT effectively reduces LDLc and increases HDLc in hypercholesterolemic postmenopausal women with CHD. The effects on LDLc and HDLc concentrations in these patients with a high risk for new complications are comparable to the results reported in healthy normocholesterolemic women.[3,32]

Whether the cyclic addition of progesterone to ERT decreases the favorable effects of ERT on the risk reduction for CHD is controversial.[1,32] Grodstein et al[32] showed that the use of progestagen did not decrease the favorable effect of ERT on cardiovascular disease. We studied the effects of MPA on plasma lipids and on oxidation parameters. In accordance with the results known from the literature, the main effect of MPA in our study population was observed for the concentration of HDLc and its apolipoprotein, apo A-1. Both HDL_2 and HDL_3 were decreased by MPA. The decrease in HDL levels may be related to the observed decrease in LPL activity. We did not observe an effect of MPA on either the LDL pattern or the lag time.

Our data show that the effects of ERT on plasma lipid and estrogen levels are less pronounced in smokers than in nonsmok-

ers. This is in agreement with the data from Jensen and Christiansen,[33] who showed that smoking diminishes the decrease in LDLc on oral estrogen therapy in healthy postmenopausal women. The fact that smoking also diminishes the HDLc increase on ERT has not been described. This finding has substantial implications for cardiovascular risk reduction, as HDLc appears to be the most important risk indicator in women.[34] Plasma concentrations of estrone and estradiol were approximately twice as high in nonsmoking women on ERT versus smokers. It is likely that the diminished effects of ERT on serum lipid levels in women who smoke are related to decreased estrogen levels in these women. Estrogen concentrations in women who smoke may be lower than in nonsmoking women because smoking favors the hydroxylation of estrone at the C2 position over hydroxylation at C-16 alpha.[35] Hydroxylation at C2 leads to the formation of inactive metabolites and hence to a decreased bioavailability at estrogen target tissue. To our knowledge, no data on the possible influence of smoking on the metabolic effects of orally administered progesterone are available. In our analysis, addition of 10 mg MPA to estrogen for 14 days resulted in a 10% decrease of HDLc in nonsmokers and smokers.

Thus, HDLc was lower at baseline and increased less on ERT but showed a similar decrease on MPA in smokers compared with nonsmokers. It has been suggested that up to half of the apparent cardiovascular benefits in estrogen-treated women are due to higher HDLc levels.[3,36] Therefore, our data may have serious implications for the effectiveness of ERT in the prevention of CHD in female smokers. However, it cannot be excluded that differences in sample size between smokers (n = 10) and nonsmokers (n = 15) affected the statistical analysis, and therefore, these findings warrant further investigation.

It has been shown that smoking promotes oxidative modification of LDL in vivo.[16] Whether smoking enhances the susceptibility to in vitro oxidation of LDL particles is unclear.[18,37] Baseline data from smokers and nonsmokers in our study did not show a difference in the concentration of autoantibodies to oxidized LDL or the lag time.

We conclude that ERT may have antioxidative effects and effectively reduces LDLc and increases HDLc in hypercholesterolemic women with CHD. Therefore, ERT can be effective in the treatment of mild hypercholesterolemia in postmenopausal women with CHD, especially when they refrain from smoking.

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