

# Lipoprotein, apolipoprotein, and lipolytic enzyme changes following estrogen administration in postmenopausal women

D. Applebaum-Bowden,<sup>1,\*†</sup> P. McLean,<sup>\*</sup> A. Steinmetz,<sup>2,\*</sup> D. Fontana,<sup>\*</sup> C. Matthys,<sup>\*</sup> G. R. Warnick,<sup>\*</sup> M. Cheung,<sup>\*</sup> J. J. Albers,<sup>\*</sup> and W. R. Hazzard<sup>3,\*†</sup>

Department of Medicine,<sup>\*</sup> University of Washington School of Medicine, Seattle, WA 98195, and Department of Medicine,<sup>†</sup> Johns Hopkins University School of Medicine, Baltimore, MD 21205

**Abstract** To test whether estrogen can modulate the cholesterolemic response to an Occidental diet, six healthy postmenopausal women were studied for 84 days while ingesting a solid food diet of constant composition high in cholesterol content (995 mg/d). In the middle of the study, estrogen (17 $\alpha$ -ethinyl estradiol, 1  $\mu$ g/kg per day) was administered orally. Ingestion of the diet for the initial 28 days did not alter lipoprotein lipid or apolipoprotein (apo) levels. However, with just 4 days of estrogen use there were significant decreases in apoE (–36%), low density lipoprotein cholesterol (–26%), and postheparin plasma hepatic triglyceride lipase activity (HTGL) (–61%), and an increase in high density lipoprotein (HDL) triglyceride (72%). These changes persisted throughout the estrogen use. The percent change in HTGL with 4 days of estrogen correlated inversely with the percent change in HDL triglyceride ( $r_s = -0.94$ ). After 28 days of estrogen there were also significant increases in HDL cholesterol (21%), HDL<sub>2</sub> cholesterol (42%), apoA-I (37%), and apoA-II (9%), and a decrease in apoB (–11%). The level of apoE at this juncture correlated inversely with the level of HDL cholesterol ( $r_s = -0.90$ ), and the levels of HTGL and apoA-I correlated with HDL<sub>2</sub> cholesterol ( $r_s = -0.89$  and  $r_s = 0.89$ , respectively). Thus, HTGL may play a role in both the early estrogen-related changes in HDL triglyceride and apoE and the late estrogen-related changes in HDL cholesterol, apoA-I, and apoA-II. — Applebaum-Bowden, D., P. McLean, A. Steinmetz, D. Fontana, C. Matthys, G. R. Warnick, M. Cheung, J. J. Albers, and W. R. Hazzard. Lipoprotein, apolipoprotein, and lipolytic enzyme changes following estrogen administration in postmenopausal women. *J. Lipid Res.* 1989. 30: 1895–1906.

**Supplementary key words** apolipoprotein A-I • high density lipoprotein • hepatic triglyceride lipase • lecithin:cholesterol acyltransferase

Postmenopausal women have a greater risk of coronary heart disease than premenopausal women (even at comparable ages), but this difference disappears when oophorectomized women use exogenous estrogens (1). Mean total and low density lipoprotein (LDL) cholesterol levels are lower in premenopausal and higher in postmenopausal American women than in men of comparable age,

suggesting a role for estrogen in modulating the cholesterolemic response to an Occidental diet (2). The protective effect of estrogen use, however, may be mediated through increased high density lipoprotein (HDL) cholesterol levels (3). Despite an increase in postmenopausal estrogen replacement therapy, there is a paucity of information regarding the LDL and HDL responses. Detailed studies of the response of the major apolipoprotein in HDL, apoA-I, to estrogen use in postmenopausal women are also not available. However, studies in young premenopausal women have shown that ethinyl estradiol administration is associated with increases in HDL cholesterol levels (4–7) and apoA-I levels (4, 7). Extrapolation of these results to postmenopausal women would suggest that they, too, should have increased apoA-I levels with estrogen use. ApoE is also associated with HDL (8). The response of apoE levels to estrogen use is not known but they may be influenced by estrogens since they are influenced by gender (8, 9).

Hepatic triglyceride lipase (HTGL) (10–15) and, more clearly, lipoprotein lipase (LPL) (10, 16–18) have major roles in the removal of triglyceride from triglyceride-rich

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein; HTGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; CRC, Clinical Research Center; VLDL, very low density lipoprotein; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; LCAT, lecithin:cholesterol acyltransferase;  $r_s$ , Spearman's rank correlation coefficient; P/S, polyunsaturated to saturated.

<sup>1</sup>To whom reprint requests should be addressed at present address: Department of Internal Medicine, Bowman Gray School of Medicine of Wake Forest University, 300 South Hawthorne Road, Winston-Salem, NC 27103.

<sup>2</sup>Present address: Zentrum Innere Medizin, Endokrinologie und Metabolismus, Baldingerstrasse, Philipps Universität, D-355, Marburg, West Germany.

<sup>3</sup>Present address: Department of Internal Medicine Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103.

lipoproteins and in the formation of HDL. The response of these enzymes in postmenopausal women given ethinyl estradiol has not been reported. If postmenopausal women respond like premenopausal women given ethinyl estradiol, HTGL activity should decrease with no change in LPL activity (5, 7). Given the proposed role of HTGL in lipoprotein metabolism, information regarding the temporal relationships between the decrease in HTGL and the changes in the lipoproteins and apolipoproteins associated with ethinyl estradiol administration would yield important information.

The present study was designed to determine whether ethinyl estradiol administration to normolipidemic postmenopausal women could modulate lipoprotein metabolism during a high cholesterol diet. The study had three principal aims: 1) to determine the timing of their lipoprotein lipid and apolipoprotein responses; 2) to determine the timing of their HTGL responses, and 3) to determine the temporal (and, possibly by implication, mechanistic) relationship between these changes.

## METHODS

### Subjects

Nonsmoking postmenopausal women who were not taking any medication known to influence lipid metabolism were recruited. Two to 3 weeks prior to beginning the study, the volunteers had overnight fasting blood samples drawn to determine lipoprotein lipid levels and tests of liver, kidney, and thyroid function. Only healthy women who were below the 90th percentile of the Lipid Research Clinic's Reference values for white females of similar age not taking hormones were accepted into the study (19). The subjects selected had their last menstrual period 4 to 20 years prior to participation in this study. During the study, occasional use of aspirin was reported by subject #1, chlorthalidone by subject #2, imipramine by subject #3, and acetaminophen by subject #6. Subject #3 also took dicloxacillin from day 36 to day 45.

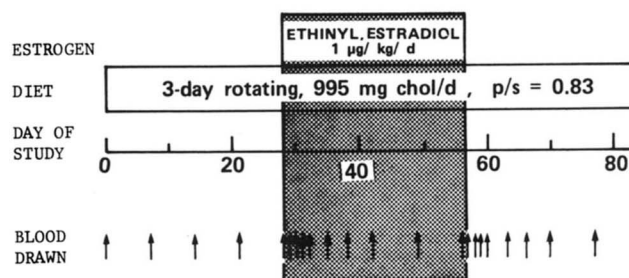
### Experimental design

The diet used in this study was the high cholesterol diet phase described earlier by Applebaum-Bowden et al. (20). The basic diet plan was a 3-day rotating schedule, with each day's diet containing 45% of the calories as carbohydrate, 40% as fat ( $P/S = 0.8$ ), and 15% as protein. The six women were interviewed individually by the dietitian at the Clinical Research Center (CRC) to estimate their energy expenditure and hence isocaloric food requirement. The calculation of the total calories needed per day to maintain their body weights took into consideration the subject's basal metabolic rate (21) (awake and asleep, age-adjusted), physical activity, and specific dynamic action (20). In addition, the subjects were instructed to keep a 3-

day food record. This ad libitum diet information was used to determine whether any subjects would experience major dietary changes going from their ad libitum diet to the study diet and as a second estimate of their energy expenditure. Prior to initiating the study, the diet (excluding the fruit, tomato juice, and chocolate pudding) for the entire 84 days of the study for each subject was prepared using her calculated caloric requirements. The subjects were instructed to eat only the food provided, and all of the subjects' meals were prepared by the CRC metabolic kitchen. The subjects were instructed to maintain the same level of physical activity throughout the study. No alcohol or caffeine-containing beverages were consumed. Because of the complexity of the food preparation and storage requirements, a maximum of two subjects were studied at the same time.

The study consisted of three phases (Fig. 1). The first phase lasted 28 days, during which the subjects consumed the diet without estrogen supplementation. In the second phase the volunteers took 17 $\alpha$ -ethinyl estradiol (Schering Estinyl Estradiol, 1  $\mu\text{g}/\text{kg}$  per day, to the nearest 10 kg weight using combinations of 0.05 and 0.02 mg tablets, average dose 0.07 mg/day) for 28 days while continuing the constant diet. Finally, during the third phase, the subjects stopped taking the estrogen but continued on the diet for another 28 days. The protocol was approved by the University of Washington Human Subjects Committee. All subjects gave informed written consent.

Subjects were seen on an outpatient basis. All blood samples were drawn after subjects had fasted overnight (12–14 h), on the day the study began, day 0, and on days 7, 14, 21, 28–32, 35, 38, 42, 49, 56–60, 63, 66, 70, 77, and 84. Antecubital venous blood was drawn through a 21-gauge butterfly infusion kit and mixed immediately with dry disodium EDTA (1.5 mg/ml blood) in a Vacutainer tube (Becton Dickinson and Co, Rutherford, NJ). Beginning with day 14 heparin (10 U/kg; Upjohn Company, Kalamazoo, MI) was injected after the above blood samples were obtained, followed by 10 ml of 0.9% sterile saline to flush the line. Blood was drawn 10 min after the



**Fig. 1.** Study protocol. The diet consumption is indicated by the long rectangle from day 0 to day 84. The administration of ethinyl estradiol from day 28 to day 56 is indicated by the shaded area. The blood samples (fasting and beginning with day 14, 10 min after the heparin injection) were drawn at the times indicated by the arrows.



heparin injection, mixed with EDTA in a similar Vacutainer tube, and placed in ice. For all blood samples centrifugation was performed at 1500 g for 30 min at 4°C and the resulting plasma was collected.

### Lipid and lipoprotein analysis

HDL cholesterol and triglyceride were measured after dextran sulfate (50,000 daltons)-MgCl<sub>2</sub> precipitation of the apoB-containing lipoproteins from plasma (22). The plasma samples collected during the baseline and on days 0, 28, 56, and 84 of the study were fractionated by ultracentrifugation at d 1.006 g/ml. Very low density lipoprotein (VLDL) cholesterol was calculated as total cholesterol minus the infranantant cholesterol; LDL cholesterol was calculated as the d 1.006 g/ml infranantant cholesterol minus HDL cholesterol (23). On the remaining days LDL cholesterol was estimated by subtracting HDL cholesterol and VLDL cholesterol (determined on days 0, 28, 56, and 84 and interpolated for the days between measurements) from the total plasma cholesterol level. HDL subfractions were measured by the method of Warnick, Benderson, and Albers (24) as described in Bachorik and Albers (25). Cholesterol and triglyceride were analyzed by standard Lipid Research Clinic methodology with the AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, NY) (23).

### Apolipoprotein analysis

Plasma samples for determination of apoA-I, A-II, B, D, and E were supplemented with sodium azide (0.05%), chloramphenicol (0.001%), and gentamicin (0.0005%). The samples were frozen in sealed 1-ml vials (Wheaton Scientific, Millville, NJ) and stored at -20°C. ApoA-I, A-II, and D were measured by radial immunodiffusion assays (4, 26, 27). ApoB was measured by radioimmunoassay (28). ApoE was measured by radioimmunoassay as described below.

VLDL was isolated from three hyperlipidemic subjects, each of whom was homozygous for one of the three apoE isoforms. ApoE was purified from VLDL by preparative polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) (29, 30). The fractions containing only apoE (by analytical polyacrylamide gel electrophoresis in SDS) were dialyzed against 0.02 M ethylmorpholine (pH 8.6) followed by 5 mM NH<sub>4</sub>HCO<sub>3</sub>, lyophilized, and stored at -70°C for use within 4 months. For injection, apoE (200 µg per rabbit, determined using bovine serum albumin [BSA] in 0.1% SDS as the protein standard (31)) was subjected to isoelectric focusing in cylindrical gels (32). One gel was stained with Coomassie Brilliant Blue G250 to locate the apoE isoforms. The sections from the other gels corresponding to apoE were excised, emulsified with complete Freund's adjuvant (Difco Laboratories, Detroit, MI), and injected subcutaneously into New

Zealand white rabbits. Booster injections were prepared similarly using incomplete Freund's adjuvant and given 7, 14, and 30 days after the initial injection. Each of the four antisera produced (one for each isoform and a mixture of the three isoforms) gave a single precipitin line with the purified apoE and with plasma from each of the three homozygous subjects, but did not react with apoA-I, A-II, C-II, and C-III. The antiserum produced against the mixture of the apoE isoforms was used in the radioimmunoassay described below.

ApoE was labeled with <sup>125</sup>I by the iodine monochloride method (33, 34). Lyophilized apoE (100 µg) was dissolved in 50–100 µl of 0.5 M glycine buffer (pH 10.0, containing 0.5 M NaCl). Carrier free Na<sup>125</sup>I (1 mCi in 10 µl) was added with rapid stirring followed by 10 µg ICl (1 µg/µl in 0.1 N HCl). After 2 min of stirring, the mixture was dialyzed against buffer A (0.1 M sodium phosphate buffer, pH 7.4, containing 0.01 M sodium decyl sulfate and 0.02% sodium azide) supplemented with 20 mM KI. After the second change of buffer, 100 µl of 6% BSA was added to the labeled apoprotein. The <sup>125</sup>I-labeled apoE was purified by chromatography on Sephadex G-150 equilibrated with 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1 M sodium decyl sulfate, 1% BSA, and 0.02% sodium azide, dialyzed against buffer B (buffer A supplemented with 0.1% Tween 20), and stored at 4°C. The specific activity of the <sup>125</sup>I-labeled apoE was generally 2000 cpm/ng protein. Less than 1% of <sup>125</sup>I was soluble in 10% trichloroacetic acid.

Each assay tube (10 × 75 mm glass tubes precoated with 1% column coat [Miles Laboratories, Elkhart, IN]) contained 100 µl of <sup>125</sup>I-labeled apoE (1 µg/ml); 100 µl of buffer B, plasma sample (1:50) or standard (0.1 to 4 µg/ml of purified apoE) diluted with buffer B; and 100 µl of rabbit anti-apoE serum diluted with buffer C (0.1 M sodium phosphate buffer, pH 7.4, containing 1% BSA and 0.02% sodium azide). After incubation for 24 h (4°C), 100 µl of normal rabbit serum (1:100) and 300 µl of goat anti-rabbit IgG (1:3) diluted with buffer C were added sequentially. The assay tubes were incubated overnight (4°C), and centrifuged (2000 g, 25 min, 4°C). The immunoprecipitates were washed three times with 1 ml of buffer C and the remaining radioactivity was measured. Nonspecific precipitation was estimated by substituting buffer B for the anti-apoE serum. All samples were assayed in triplicate.

Assay data were calculated using a logit transformation program (Tektronix, Inc., Beaverton, OR). The correlation coefficient (*r*) of the logit of B/Bo versus log antigen dose averaged -0.993 and the slope -1.87 ± 0.27 (mean ± SD) (*n* = 23). The between-assay reproducibility assessed by analyzing samples from three frozen plasma pools each assay day ranged from 5 to 11% for most plasma samples. The apoE concentration (mean ± SD) in the pools was 5.63 ± 0.40 mg/dl, 4.71 ± 0.24 mg/dl, and 3.12 ± 0.36 mg/dl.

Optimal sensitivity was obtained with 0.01 M sodium decyl sulfate and 0.1% Tween 20 in the assay buffer. Other conditions tested were preheating plasma at 37°C for 1 h, delipidation with Triton X-100, addition of 4–8 M urea, and pretreatment of plasma with tetramethylurea. Under these assay conditions, plasma and isolated lipoprotein fractions (VLDL, LDL, HDL<sub>2</sub>, HDL<sub>3</sub>) yielded displacement curves that paralleled the curves produced by the apoE standard. When purified apoE was added to plasma, the predicted increase in apoE concentration was obtained. Repeated freezing and thawing of samples did not affect the measured values. Purified apoA-I, A-II, or D did not displace apoE.

#### Triglyceride lipases and lecithin:cholesterol acyltransferase (LCAT) mass assays

The postheparin plasma was collected, frozen, and stored at –20°C. Within 2 months following the last clinic visit the postheparin plasma was thawed and assayed for HTGL activity as described by Applebaum-Bowden et al. (15). The substrate contained, per ml, trioleoyl glycerol (2.9 μmol), glyceryl tri[1-<sup>14</sup>C]oleoyl (0.13 μCi), gum arabic (10 mg), BSA (20 mg), and 2 M NaCl (0.5 ml) (16). The assays were run at 28°C for 90 min and the released <sup>14</sup>C-labeled fatty acids were extracted (35). LPL activity was calculated by subtracting the HTGL activity from the total triglyceride lipase activity, which was measured using a substrate that contained 0.1 ml serum and 0.2 M NaCl (instead of 2 M NaCl). The amount of endogenous triglyceride added to the assay in the postheparin plasma sample did not decrease the observed activity (15, 36).

Preheparin plasma that had been stored as described for apolipoprotein assays was used to measure LCAT mass by radioimmunoassay (37).

#### Data analysis

The results are presented as means ± SD. The data were analyzed by the paired *t* statistic and by the non-parametric Spearman's rank correlation coefficient (*r*<sub>s</sub>) (38).

## RESULTS

#### Baseline information

The six postmenopausal women who volunteered for this study varied in age from 53 to 67 yr (mean 57 yr) and in body mass index from 24.2 to 32.2 kg/m<sup>2</sup> (mean 27.9) (Table 1). Their ad libitum diets contained 279 ± 92 (mean ± SD) mg of cholesterol per day and had a polyunsaturated to saturated (P/S) fatty acid ratio of 0.69 ± 0.24. When examined 2–3 weeks prior to beginning the study (while consuming their ad libitum diets), the subjects had total plasma triglyceride levels of 125 ± 35 mg/dl and total plasma cholesterol levels of 241 ± 25 mg/dl with LDL cholesterol levels of 157 ± 26 mg/dl and HDL cholesterol levels of 67 ± 11 mg/dl (Table 1).

#### Lipid, lipoprotein, and apoprotein responses to the diet

The study diet for the six subjects had a cholesterol content of 995 ± 12 mg per day and a P/S ratio of 0.83 ± 0.01. The mean weight of the subjects decreased slightly during the first phase of the study (74.5 ± 9.8 kg to 73.1 ± 10 kg, *P* < 0.05), but did not change during either of the following 28-day periods. Consuming the high cholesterol diet for 28 days did not significantly alter total plasma triglyceride (mean difference, –14 ± 22 mg/dl), the triglyceride content in the lipoprotein frac-

TABLE 1. Clinical characteristics and lipoprotein lipid levels of the postmenopausal women while on their ad libitum diets

Subject	Age	BMI	Cholesterol				Triglyceride			
			Total	VLDL	LDL	HDL	Total	VLDL	LDL	HDL
	yr	kg/m <sup>2</sup>	mg/dl							
1	53	30.6	225	30	142	53	148	87	43	18
2	54	27.7	215	20	119	77	116	79	26	11
3	54	24.2	241	8	159	75	61	25	26	10
4	56	27.8	262	14	189	59	122	78	33	11
5	58	32.2	222	13	150	59	148	111	24	13
6	67	24.7	278	22	180	76	156	105	39	12
Mean	57	27.9	241	18	157	67	125	81	32	13
SD	5	3.2	25	8	26	11	35	31	8	3

Abbreviations: BMI, body mass index; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

TABLE 2. Triglyceride and cholesterol levels in the plasma and lipoprotein fractions of the postmenopausal women the day they began the study (before the diet) and during the diet with and without estrogen

	Treatment (Day of Study)			
	None (0)	Diet (28)	Diet + Estrogen (56)	Diet (84)
	<i>mg/dl</i>			
<b>Triglyceride</b>				
Total	112 ± 35	98 ± 41	154 ± 43**	88 ± 40*
VLDL	66 ± 31	57 ± 39	89 ± 44**	46 ± 39*
LDL	33 ± 5	30 ± 8	41 ± 10**	30 ± 9**
HDL	13 ± 4	12 ± 2	24 ± 5**	12 ± 5****
<b>Cholesterol</b>				
Total	256 ± 36	255 ± 35	225 ± 30***	256 ± 41****
VLDL	14 ± 6	14 ± 4	13 ± 5	12 ± 8
LDL	172 ± 30	173 ± 22	130 ± 23**	169 ± 27**
HDL	70 ± 8	68 ± 16	82 ± 20**	76 ± 18
HDL <sub>2</sub>	33 ± 6	33 ± 11	46 ± 17****	37 ± 13
HDL <sub>3</sub>	37 ± 3	35 ± 6	36 ± 7	39 ± 6

Values given as mean ± SD, n = 6.  
Paired statistic for each period versus the previous period: \*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.02$ ; \*\*\*\*,  $P < 0.05$ .

tions, total plasma cholesterol (mean difference,  $-1 \pm 24$  mg/dl), or the cholesterol content in the lipoprotein fractions (Table 2). The mean plasma levels of apoA-I, A-II, B, E, and D also did not change in response to consuming the high cholesterol diet for 28 days (Table 3).

### Time course of the plasma and lipoprotein lipid changes in response to ethinyl estradiol

Blood was drawn from the fasting subjects frequently during the first 2 weeks of ethinyl estradiol administration, and again when the estrogen was discontinued (Fig. 1). With the administration of estrogen, there was a 15% increase in total plasma triglyceride levels after 2 days

( $P < 0.05$ ), a 36% increase after 4 days ( $P < 0.01$ ), and a continued increase that appeared to plateau after 10 days (54% increase;  $P < 0.05$ ) (Fig. 2). HDL triglyceride levels also increased almost linearly with a 36% increase after 2 days ( $P < 0.05$ ), a 72% increase after 4 days ( $P < 0.01$ ), and a continued increase that plateaued at 112% after 10 days ( $P < 0.001$ ). VLDL and LDL triglyceride were measured only at the end of each phase of the study. After 28 days of estrogen, total plasma triglyceride levels were elevated 67% (mean increase,  $56 \pm 20$  mg/dl;  $P < 0.01$ ) (Table 2). Although each lipoprotein fraction increased, the amount differed: HDL triglyceride levels increased 117% (mean increase,  $12 \pm 6$  mg/dl;  $P < 0.01$ ), VLDL triglyceride levels increased 86% (mean increase,  $33 \pm 14$

TABLE 3. Plasma apolipoprotein concentrations of the postmenopausal women the day they began the study (before the diet) and during the diet with and without estrogen

	Treatment (Day of Study)			
	None (0)	Diet (28)	Diet + Estrogen (56)	Diet (84)
	<i>mg/dl</i>			
ApoA-I	152 ± 12	155 ± 28	212 ± 38*	151 ± 20**
ApoA-II	36.4 ± 3.2	34.5 ± 3.2	37.5 ± 1.3****	35.4 ± 3.2
ApoB	126 ± 13	125 ± 15	111 ± 23****	132 ± 20****
ApoE	6.94 ± 1.76	6.84 ± 1.43	4.60 ± 0.95**	7.21 ± 1.24***
ApoD	7.27 ± 0.38	7.33 ± 1.02	7.38 ± 0.77	7.30 ± 0.64

Values given as mean ± SD, n = 6.  
Paired statistic for each period versus the previous period: \*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.02$ ; \*\*\*\*,  $P < 0.05$ .



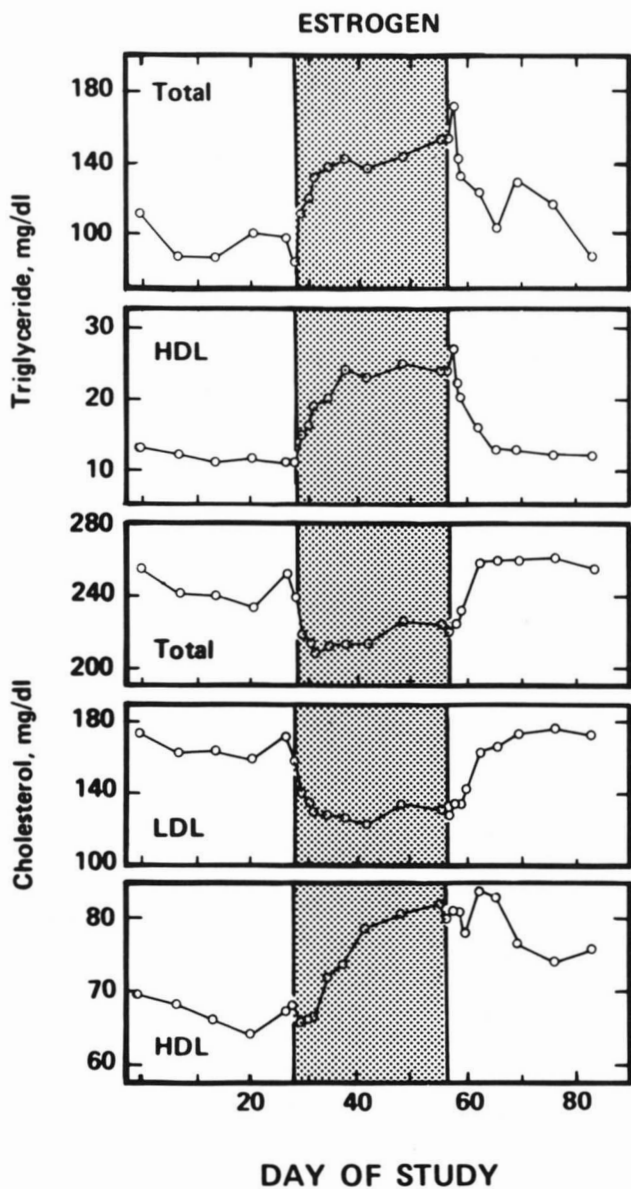


Fig. 2. Mean changes in total plasma and HDL triglyceride levels and total plasma, LDL, and HDL cholesterol levels in the six postmenopausal women. The ethinyl estradiol administration ( $1 \mu\text{g}/\text{kg}$  per day) is indicated by the shaded area from day 28 to day 56.

mg/dl;  $P < 0.01$ ), and LDL triglyceride levels increased 37% (mean increase,  $11 \pm 4$  mg/dl;  $P < 0.01$ ).

With the estrogen administration there was a 15% decrease in total plasma cholesterol levels after 2 days ( $P < 0.001$ ) (Fig. 2), an 18% decrease after 4 days ( $P < 0.001$ ), but only an 11% decrease after 28 days (mean decrease,  $30 \pm 19$  mg/dl;  $P < 0.02$ ) (Table 2). The decreases in total plasma cholesterol levels reflected differential changes in the various lipoprotein fractions. LDL cholesterol levels decreased 20% after 2 days ( $P < 0.001$ ) and remained decreased throughout the estrogen administration. In contrast, HDL cholesterol levels

were not altered by 4 days of estrogen, but were increased 9% after 10 days of estrogen ( $P < 0.02$ ), and continued to increase (though approaching a plateau by day 14) during the estrogen. Whereas total HDL cholesterol levels did not change early during estrogen, HDL<sub>2</sub> cholesterol levels were elevated ( $P < 0.01$ ) and HDL<sub>3</sub> cholesterol levels decreased ( $P < 0.01$ ) after 2 days (Fig. 3). Both HDL subfractions returned to the pre-estrogen levels after 4 days of estrogen. Thereafter, the increase in total HDL cholesterol was attributable specifically to increases in HDL<sub>2</sub> cholesterol. HDL<sub>2</sub> cholesterol levels increased 25% after 10 days ( $P < 0.05$ ), and continued to increase (albeit at a slower rate) throughout the estrogen administration. After 28 days of estrogen, LDL cholesterol levels were decreased 25% (mean decrease,  $43 \pm 22$  mg/dl;  $P < 0.01$ ), HDL cholesterol levels increased 21% (mean increase,  $14 \pm 6$  mg/dl;  $P < 0.01$ ), and HDL<sub>2</sub> cholesterol levels increased 42% (mean increase,  $14 \pm 11$  mg/dl;  $P < 0.05$ ), with no significant change in VLDL or HDL<sub>3</sub> cholesterol levels (Table 2). Thus, when the results from the end of the diet alone phase of the study were compared with those after 28 days of estrogen, the LDL cholesterol:HDL cholesterol ratio decreased from 2.63 to 1.67 ( $P < 0.001$ ) and the cholesterol:triglyceride ratio decreased in VLDL (0.27 to 0.15,  $P < 0.01$ ), LDL (5.56 to 3.23,  $P < 0.001$ ), and HDL (5.56 to 3.33,  $P < 0.05$ ).

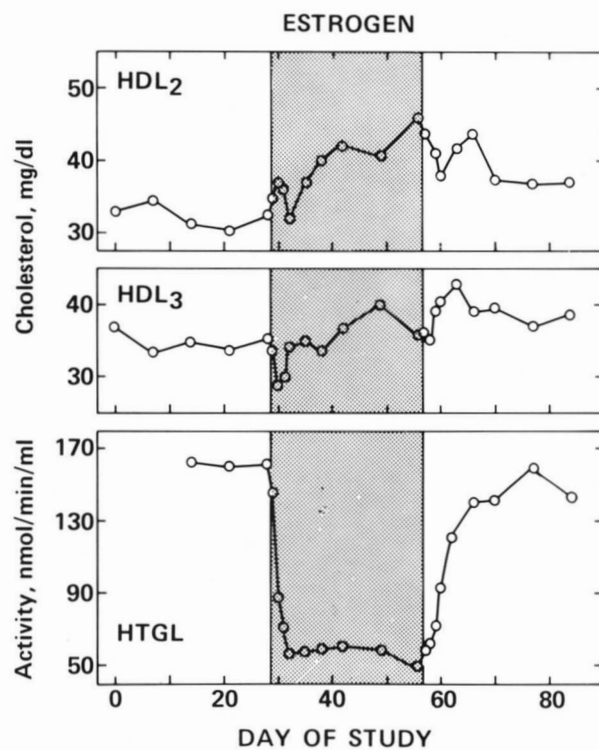


Fig. 3. Mean changes in HDL<sub>2</sub> and HDL<sub>3</sub> cholesterol levels and postheparin plasma HTGL activity in the six postmenopausal women. The ethinyl estradiol administration ( $1 \mu\text{g}/\text{kg}$  per day) is indicated by the shaded area from day 28 to 56.

When the estrogen was discontinued, the total plasma and HDL triglyceride levels decreased to the pre-estrogen (diet alone) levels after 10 days (Fig. 2). The total plasma and LDL cholesterol levels increased to the pre-estrogen levels within 7 days. HDL cholesterol levels remained elevated for 10 days after discontinuation of the estrogen and then decreased, but by the end of the study (after 28 days) they remained 12% elevated (day 28 vs day 84,  $P < 0.01$ ). The decrease in HDL<sub>2</sub> cholesterol levels paralleled the total HDL cholesterol levels, but the HDL<sub>3</sub> cholesterol levels increased 22% 7 days after discontinuation of the estrogen ( $P < 0.05$ ) (Fig. 3).

#### Time course of the apolipoprotein changes in response to ethinyl estradiol

Plasma apoA-I levels were 20% increased after 7 days of ethinyl estradiol ( $P < 0.02$ ), and 30% increased after 21 days ( $P < 0.001$ ) (Fig. 4). In contrast, plasma apoE levels were decreased 28% after 3 days of estrogen administration ( $P < 0.01$ ), and remained at this low level throughout the estrogen administration. Neither plasma apoA-II nor plasma apoB levels changed significantly until the estrogen had been administered for 28 days (though earlier

trends for both were suggestive). After 28 days of estrogen administration plasma apoA-I levels were 37% increased (mean increase  $57 \pm 12$  mg/dl,  $P < 0.001$ ), plasma apoE levels were decreased 31% (mean decrease  $2.23 \pm 1.26$  mg/dl,  $P < 0.01$ ), plasma apoA-II levels were increased 9% (mean increase  $2.9 \pm 2.4$  mg/dl,  $P < 0.05$ ), and plasma apoB levels decreased 11% (mean decrease  $13 \pm 12$  mg/dl,  $P < 0.05$ ) (Table 3). There was no significant change in plasma apoD levels. When the results from the end of the diet alone phase of the study were compared with those after 28 days of estrogen, the mean plasma apoA-I:A-II mass ratio increased from  $4.48 \pm 0.79$  to  $5.64 \pm 1.01$  ( $P < 0.001$ ), the mean plasma apoB:E mass ratio increased from  $18.06 \pm 4.01$  to  $24.83 \pm 6.27$  ( $P < 0.02$ ), and the mean LDL cholesterol:plasma apoB ratio decreased from  $1.40 \pm 0.17$  to  $1.18 \pm 0.15$  ( $P = 0.03$ ).

Plasma apoA-I levels decreased both immediately and progressively after discontinuing the estrogen (Fig. 4). Plasma apoA-II levels, however, continued to increase after discontinuation of the estrogen so that they were maximally elevated (16%) after 7 days off the hormone ( $P < 0.01$ ). Thereafter, the apoA-II levels decreased to the pre-estrogen levels. Plasma apoE levels remained depressed for 4 days after discontinuation of the estrogen, but returned to the pre-estrogen levels after 10 days. Plasma apoB levels were not significantly different from the pre-estrogen levels after 2 days. Thus, 28 days after discontinuation of the estrogen plasma apoA-I, A-II, B, and E levels had returned to their pre-estrogen levels (Table 3).

#### Time course of the HTGL changes

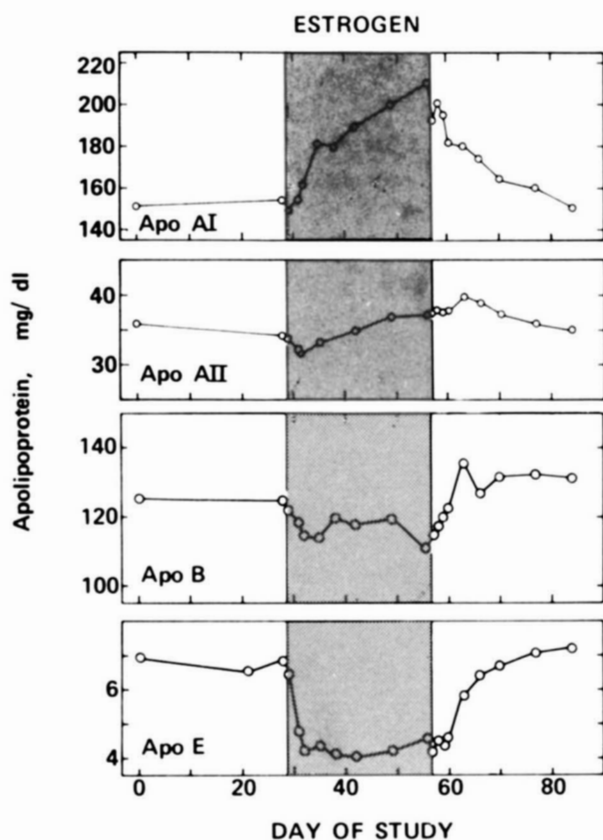
The activity of postheparin plasma HTGL was similar for the last 2 weeks on the diet alone. After the first 2 days of ethinyl estradiol administration, however, the activity decreased dramatically ( $-46\%$ ,  $P < 0.01$ ), and was further decreased ( $-61\%$ ,  $P < 0.01$ ) after 4 days (Fig. 3). This low level of activity persisted as long as the estrogen was administered so that HTGL was decreased 62% after 28 days of estrogen (mean decrease  $106 \pm 62$  nmol/min per ml,  $P < 0.01$ ) (Table 4). When the estrogen was discontinued, HTGL activity increased slowly and reached the pre-estrogen (diet alone) level ( $160 \pm 49$  nmol/min per ml) after 21 days.

#### LPL activity and LCAT mass (Table 4)

Postheparin plasma LPL levels were not significantly altered by the estrogen administration. The mean LCAT mass did not change significantly in response to the diet or the estrogen administration.

#### Relationships among the changes

Although the correlation coefficients for the relationships given below are all  $> 0.886$  for  $P < 0.05$ , one needs to



**Fig. 4.** Mean changes in total plasma apolipoprotein A-I, A-II, B, and E levels in the six postmenopausal women. The ethinyl estradiol administration ( $1 \mu\text{g}/\text{kg}$  per day) is indicated by the shaded area from day 28 to day 56.



TABLE 4. Postheparin plasma triglyceride lipase activities and LCAT mass of the postmenopausal women the day they began the study (before the diet) and during the diet with and without estrogen

	Treatment (Day of Study)			
	None (0)	Diet (28)	Diet + Estrogen (56)	Diet (84)
	<i>nmol/min/ml</i>			
HTGL	ND	162 ± 55	51 ± 27*	144 ± 26*
LPL	ND	125 ± 99	94 ± 72	101 ± 48
	<i>µg/ml</i>			
LCAT	5.34 ± 0.74	5.37 ± 0.69	5.11 ± 0.60	5.15 ± 0.52

Values given as mean ± SD; HTGL and LCAT: n = 6; LPL: n = 5; ND, not determined. Paired statistic for each period versus the previous period: \*,  $P < 0.01$ .

keep in mind that there is likely to be a  $P < 0.05$  result just by chance given the number of relationships tested.

ApoE associates with VLDL and HDL. However, at the end of the first phase (diet alone) plasma apoE levels were not correlated with either total, VLDL, or HDL triglyceride levels, or with HDL or HDL<sub>2</sub> cholesterol levels. After 28 days of estrogen, plasma apoE levels were inversely correlated with HDL cholesterol levels ( $r_s = -0.90$ ,  $P < 0.05$ ), but were not correlated with total, VLDL, or HDL triglyceride levels, or with HDL<sub>2</sub> cholesterol levels.

At the end of phase one, the diet alone, plasma apoA-I levels were correlated with HDL<sub>3</sub> cholesterol levels ( $r_s = 0.96$ ,  $P < 0.05$ ), but not HDL<sub>2</sub> cholesterol levels. After 28 days of estrogen, apoA-I levels were increased 37% similar to the 42% increase in HDL<sub>2</sub> cholesterol levels, and two were correlated ( $r_s = 0.89$ ,  $P < 0.05$ ). ApoA-I did not correlate with HDL<sub>3</sub> cholesterol levels at this time.

Postheparin plasma HTGL levels at the end of the first phase were not correlated significantly with HDL<sub>2</sub>

cholesterol ( $r_s = -0.26$ ) or VLDL triglyceride ( $r_s = 0.07$ ) levels. Since HTGL levels decreased before any change in HDL<sub>2</sub> cholesterol levels but in a similar time frame as HDL triglyceride levels, we examined whether these changes were correlated. The percent change in HTGL after only 4 days of estrogen was closely correlated with the percent change in HDL triglyceride ( $r_s = -0.94$ ,  $P < 0.05$ ). After 28 days of estrogen administration, postheparin plasma HTGL levels were significantly correlated with HDL<sub>2</sub> cholesterol levels ( $r_s = -0.89$ ,  $P < 0.05$ ) (Fig. 5), but no longer with HDL triglyceride levels.

## DISCUSSION

Epidemiological evidence suggests that estrogen may play a role in modulating the cholesterolemic response to an Occidental diet (2). To examine this more directly, we studied the effect of estrogen administration while subjects were consuming a high cholesterol diet. The subjects in this study responded in a very consistent manner, which probably reflects the strict diet control (with no caffeine or alcohol consumption), the constancy of their physical exercise, and their nonsmoking status. The lack of a response to the high cholesterol diet is not surprising considering that we previously found that the response to the high cholesterol diet was dependent upon the subject's prior diet (20). Thus, subjects on a low cholesterol diet (137 mg cholesterol per day) before the high cholesterol diet demonstrated a clear increase in total plasma cholesterol, but those on their ad libitum diets did not. Schonfeld et al. (39) also found that adding 750 mg of cholesterol to a diet with a P/S ratio of 0.8 and containing 300 mg of cholesterol produced insignificant increases in LDL cholesterol. The increases in total plasma and lipoprotein triglyceride, HDL cholesterol, apoA-I, and the decrease in postheparin plasma HTGL experienced by these postmenopausal women with estrogen during the high cholesterol diet are similar to those observed with es-

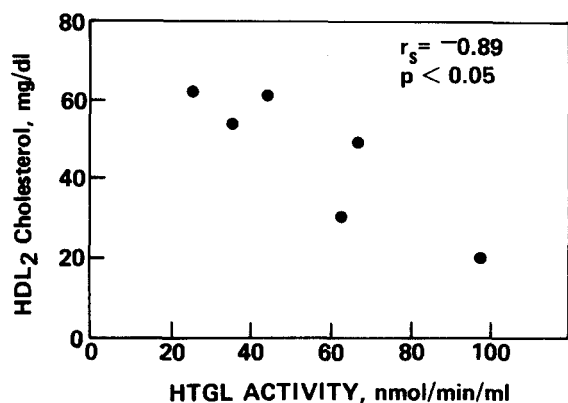


Fig. 5. The relationship between the percent change in postheparin plasma HTGL levels with 28 days of ethinyl estradiol administration (1 µg/kg per day) and the percent change in HDL<sub>2</sub> cholesterol levels during the same time period.



trogen in our previous study of premenopausal women<sup>4</sup> (4, 5) on their ad libitum diet. Thus, estrogen administration was associated with modified lipoprotein lipid, apolipoprotein, and lipolytic activity levels despite the high cholesterol diet.

The decrease in LDL cholesterol described here was not found by us or Schaefer et al. (7) with premenopausal women. The LDL response is probably related to the estrogen-deficient status of these postmenopausal women rather than the diet, since ethinyl estradiol also decreases LDL cholesterol levels in men (40). Furthermore, the 26% decrease in LDL cholesterol found in this study agrees well with the 27% reduction reported by Wallentin and Larsson-Cohn (41) in their study of ethinyl estradiol in postmenopausal women. The agreement between the results of the current study and those in the literature when subjects were consuming an ad libitum diet would imply that our results can be generalized to women in the population.

Recently we compared the effects of different estrogens in postmenopausal women and found that estrone administration was associated with a 15% decrease in LDL cholesterol whereas there was no change with 17 $\beta$ -estradiol (42). In the population-based experience of the collaborative Lipid Research Clinics Prevalence Study, conjugated equine estrogens were associated with 14% lower LDL cholesterol levels in postmenopausal women (43). Studies with estradiol valerianate have found that normolipidemic postmenopausal women did not experience a decrease in LDL cholesterol levels (44). The LDL cholesterol response, then, appears to depend both on the estrogen status of the subjects and on the estrogen administered.

The level of apoE seen in the current study of postmenopausal women is similar to the level for women age 50–64 not taking sex steroids (6.42 mg/dl) reported by Phillips, Havel, and Kane (9). The lack of change in total apoE in response to the high cholesterol diet seen in this study confirms our previous observations (45) and those of others (39, 46). Although Phillips et al. (9) reported a trend toward higher apoE levels in women, they found 8% lower serum apoE levels in women age 45–64 taking estrogens than women of similar age not using estrogens. The difference, however, was not significant. The latter finding is consistent with the significant decrease in apoE levels in response to estrogen found in the current study. The variation in the magnitude of the responses may relate to the estrogen used similar to the LDL cholesterol responses discussed above.

<sup>4</sup>These premenopausal women experienced an increase in VLDL triglyceride (84%,  $P < 0.01$ ), LDL triglyceride (32%,  $P < 0.01$ ), and HDL triglyceride (133%,  $P < 0.001$ ) with no change in VLDL cholesterol levels with a similar dose of ethinyl estradiol for 14 days (Applebaum-Bowden, D., et al., unpublished observations).

In addition to the decrease in total plasma apoE, there is reason to believe that there was a change in the distribution of apoE in the lipoprotein fractions in response to estrogen. If hydrolysis of triglyceride-rich lipoproteins by HTGL mediates a preferential transfer of apoE to large HDL as has been suggested by Rubinstein et al. (47), the decrease in HTGL with estrogen may result in less apoE being transferred to large HDL. This idea is supported by the finding by Phillips et al. (9) that apoE constitutes the same percent of VLDL protein (tetramethylurea-soluble) in normotriglyceridemic women with or without estrogen treatment. Together, these results suggest that the percent of apoE associated with VLDL should increase during estrogen administration, leading to a decrease in the amount of apoE associated with HDL. The decrease in the concentration of apoE during estrogen coupled with this proposed alteration in the distribution of apoE may well have resulted in a dramatic decrease in the apoE-containing lipoproteins of molecular weight similar to HDL.

Estrogen increases the level of the apoB/E receptor in rat liver (48, 49) and appears to result in more binding to hepatocytes judging from cell-associated <sup>125</sup>I-labeled LDL (50–52). If humans behave similarly, estrogens should increase the apoB/E receptor activity in liver facilitating the removal of both apoB- and apoE-containing lipoproteins. High dietary cholesterol appears to down-regulate the LDL receptor in humans (20, 53). It is not clear whether estrogen could overcome this inhibition so that the LDL receptor would be increased. Increasing the LDL receptor is an attractive hypothesis, however, in that both apoB and apoE decreased with estrogen. Our observed lack of a major decrease in the plasma apoB concentration with estrogen might suggest that the apoE-containing lipoproteins were selectively removed, that the synthesis of apoB was increased, or both. Also, it is conceivable that estrogen specifically enhanced an apoE-only receptor or that the decrease in apoE with estrogens may be related to altered synthesis rather than to enhanced removal. Further studies will be necessary to understand the differences in the metabolism of apoE and B induced with estrogen.

The current study of estrogen administration has provided an opportunity to examine the temporal changes of specific lipoprotein fractions. LDL and HDL changed in different directions and at different rates. After 3 days of estrogen administration, LDL cholesterol decreased 23% with no change in plasma apoB levels. Therefore, the decrease in the LDL cholesterol:plasma apoB ratio we reported after 28 days actually occurred after only 3 days and suggests a change in composition rather than particle number. ApoA-I, the main protein in HDL, increased after 7 days of estrogen without a concomitant increase in apoA-II, so the apoA-I:apoA-II ratio increase occurred after 7 days. The percentage of plasma apoA-I lipoprotein

particles with no apoA-II is highly correlated with the apoA-I:apoA-II molar ratio (54). Using that correlation and the molar ratio, we calculate that 32% of plasma apoA-I was not associated with apoA-II at the end of the initial phase (diet alone), but after 7 days of estrogen this increased to 42%, possibly a 31% increase in particles containing apoA-I with no apoA-II. There were dramatic changes in HDL triglyceride and HTGL, but no change in HDL cholesterol after 4 days of estrogen, so that the HDL cholesterol:HDL triglyceride ratio decreased. The rapid increase in HDL triglyceride is what would be expected if there were a precursor-product relationship between VLDL triglyceride and the triglyceride in an HDL subfraction (55). The correlation between the percent change in HTGL and the percent change in HDL triglyceride levels suggests that the enzyme may play a role in the early changes in HDL in response to estrogen. The later inverse relationship between HTGL activity and HDL<sub>2</sub> cholesterol levels suggests a different mechanism for this correlation, perhaps, as proposed by Bamberger, Glick, and Rothblat (56) reflecting reduced HDL catabolism mediated by the phospholipase activity of this hepatic (endothelial) lipase.

In summary, we have established that estrogen use in postmenopausal women is associated with major decreases in apoE, LDL cholesterol and HTGL activity and increases in apoA-I, HDL cholesterol, and lipoprotein triglyceride. Only HDL cholesterol levels increased gradually and remained elevated after estrogen was discontinued. The persistence of the estrogen effect may explain the observation that the protective effect of estrogen use appears to be mediated through HDL cholesterol levels (3). ■

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