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tracer kinetics

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Abstract Estrogen treatment raises plasma high density lipoprotein (HDL) levels, which may reduce cardiovascular risk. To identify the responsible mechanisms as well as the importance of the route of administration, we treated eight healthy postmenopausal women in a double-blind crossover study with three treatments for 6 weeks each: oral estradiol, 2 mg daily; transdermal estradiol, 0.1 mg twice weekly; and placebo. At the end of each treatment, apoA-I of HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) was endogenously labeled by a constant intravenous infusion of trideuterated leucine. HDL_2 and HDL_3 were separated by preparative ultracentrifugation. The pool sizes and enrichment curves of HDL apoA-I were used to calculate production rates and fractional catabolic rates (FCR). Oral estradiol increased the levels of HDL_2 apoA-I by 37% ($P < 0.005$) and of HDL_3 apoA-I by 11% ($P < 0.05$). These increased apoA-I levels resulted entirely from increased production, by 36% for HDL_2 ($P < 0.01$), and by 19% for HDL_3, ($P < 0.05$) as their FCRs were unchanged (0.20 pool/d with placebo and 0.21 with estradiol for HDL_2, and 0.19 with placebo and 0.21 with estradiol for HDL_3). The isotopic enrichment curves of HDL_2 apoA-I and HDL_3 apoA-I were identical, implying that apoA-I rapidly cycles between HDL particles, or that rapid interconversion of these subfractions occurs. The changes in HDL apoA-I metabolic rates were positively correlated with changes in VLDL-apoB metabolic rates measured previously. Transdermal estradiol, with systemic potency similar to that of oral estradiol, had no significant effect on HDL levels or metabolic rates. Thus, the "first pass" effect of oral estradiol on the liver and/or intestine appears to increase HDL apoA-I levels (particularly in HDL_2) by increasing HDL apoA-I production, and not by reducing HDL apoA-I catabolism.—**Walsh, B. W., H. Li, and F. M. Sacks.** Effects of postmenopausal hormone replacement with oral and transdermal estrogen on high density lipoprotein metabolism. *J. Lipid Res.* 1994. **35:** 2083–2093.

Supplementary key words estrogens • HDL • apoA-I • metabolism • hormones

Cardiovascular disease is the leading cause of death among women in industrialized countries. Postmenopausal women who take estrogens generally have lower rates of cardiovascular disease than women of a similar age who do not, (1–3) possibly because estrogen has favorable effects on plasma lipoprotein levels, which are risk factors for cardiovascular disease. Estrogen, particularly when taken orally, raises levels of high density lipoprotein (HDL) (4–6) and lowers those of low density lipoprotein (LDL) (4, 5). The effect of estrogen on HDL levels may be particularly important, as they are powerful predictor of heart disease in women (7).

The mechanism by which estrogen raises the levels of HDL is uncertain. The few studies that have attempted to identify the cause for these increased HDL levels have reached contradictory conclusions: one study (6) attributed the raised HDL levels to increased HDL production, whereas two others ascribed it to decreased HDL catabolism (8, 9). In the first study (6), a high dose of a potent synthetic estrogen, ethinyl estradiol, was administered to four young premenopausal women; the dose used, 100 μg, is approximately 20-times more potent than that of estrogens currently used for postmenopausal estrogen treatment (10) and three times the amount currently used in oral contraceptives. The investigators reinfused HDL with all of its proteins exogenously labeled. They found that ethinyl estradiol increased HDL protein levels solely by increasing the overall rate of HDL protein production, and not by changing the rate of its catabolism. There was a suggestion that apoA-I production might be increased, as, for two subjects in whom apoA-I was isolated, apoA-I levels rose due to increased production.

In the second study (8), treatment with estradiol valerate in 16 postmenopausal women decreased hepatic lipase

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; S_f, Svedberg units of flotation, negative sedimentation coefficient at d = 1.063 kg/l and 26°C; D_3-leucine, trideuterated leucine; ELISA, enzyme-linked immunosorbent assay; GC–MS, gas chromatography–mass spectroscopy; FCR, fractional catabolic rate; FSH, follicle-stimulating hormone.

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activity by 25%. This enzyme, present in hepatic endothelial cells, hydrolyzes HDL_2 phospholipids and triglycerides, which may lead to the uptake of HDL by hepatocytes. Although the investigators did not measure HDL turnovers, they inferred that estrogen may increase HDL levels by decreasing the rate of HDL catabolism, by estrogen-induced suppression of hepatic lipase activity. The third study (9) found that estrogen treatment of a healthy postmenopausal woman delayed apoA-I catabolism.

To distinguish between these two possibilities (increased HDL production vs. decreased HDL catabolism) we conducted a placebo-controlled crossover trial in healthy postmenopausal women given low doses of oral and transdermal estradiol; analysis of apoB metabolism in this cohort has been previously published (11). During each treatment, the rates of apoA-I production and catabolism in HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml) were measured by endogenously labeling apoA-I with an intravenous infusion of a nonradioactive amino acid tracer, trideuterated leucine. We administered estradiol by two different routes, to ascertain whether its effect on HDL metabolism is mediated by a selective action on hepatocytes and/or enterocytes. Estrogen given orally exposes enterocytes and hepatocytes to high concentrations of estrogen prior to dilution into the systemic circulation. In contrast, parenterally administered estrogen enters the systemic circulation directly, and lacks the "first pass" effect. One would therefore expect an oral estrogen to alter HDL metabolism more than a parenteral one, even if both had equal systemic potency. Indeed, many (11–14) but not all (15–17) studies of parenteral estradiol have found no significant changes in HDL concentrations.

METHODS

Subjects

Healthy postmenopausal women were eligible for study if they had had amenorrhea for at least 12 months and a serum follicle-stimulating hormone (FSH) level above 50 IU per liter. To eliminate factors influencing lipid metabolism, we included only women who were nonsmokers, weighed less than 150% of their ideal weight, consumed less than 28 ml of ethanol daily, did not have diabetes, were not taking medications that affect lipid metabolism, and had blood cholesterol and triglyceride concentrations below the 95th percentile for age (18).

Nine women, 41 to 64 years old, were enrolled and all completed the three treatment periods. These subjects had undergone measurement of apoB metabolism as reported previously (11). Due to an ultracentrifuge mishap involving one subject's plasma obtained during oral estradiol treatment, HDL from only eight subjects was available for apoA-I analysis for all three treatment periods. All subjects had taken estrogens during the preceding year; estrogen treatment was stopped at least 2 months before enrollment. Two of the nine subjects were surgically postmenopausal; another two had undergone hysterectomy with conservation of the ovaries. The remaining five women who had not undergone hysterectomy had endometrial biopsies to verify that they had no preexisting hyperplasia prior to enrollment. Informed consent was obtained from the subjects. This study was approved by the institutional review board of Brigham and Women's Hospital.

Protocol

This study was a fully randomized, double-blind, placebo-controlled crossover trial with three treatment periods each. All six possible sequences of treatment were used at least once and two sequences were used twice. The subjects were instructed to follow their usual diet and pattern of exercise during the study. We chose to study subjects consuming an unrestricted diet, so that our results could be applicable to the majority of postmenopausal women prescribed estrogen treatment who do not follow any particular dietary restrictions.

Oral micronized estradiol tablets (2 mg per day; Estrace, Mead Johnson, Evansville, IN), transdermal estradiol patches (0.1 mg twice a week; Estraderm, CIBA-Geigy, Summit, NJ), and placebo pills and patches were each used for 6 weeks. To induce withdrawal bleeding after each treatment, medroxyprogesterone acetate (10 mg per day) was given for 7 days, and then no medication was given for 2 weeks.

Measurement of lipoprotein and sex hormone concentrations

Blood samples for fasting lipoprotein measurements (cholesterol, triglyceride, and apoB in the standard lipoprotein classes) were obtained on three mornings during the last week of each treatment period, and were processed and analyzed as described below. Serum FSH was measured by radioimmunoassay (19) in pooled plasma specimens derived from 10 aliquots obtained at hourly intervals during inpatient hospitalization. Plasma estradiol and estrone levels were measured by radioimmunoassay after ether extraction and chromatography (20) in samples obtained approximately 8–10 h after a pill was taken or 24 h after a transdermal patch was applied.

Measurement of metabolic rates

At the end of each treatment period the subjects were admitted to the Clinical Research Center after a 12-h overnight fast. Estrogen and placebo pills were taken approximately 8–10 h before admission; transdermal patches were changed 24 h before admission. The subjects had recorded all foods eaten within 24 h of the first admis-

sion and had replicated this diet before each readmission. For the first 4 h at the Clinical Research Center, subjects were restricted to noncaloric and noncaffeinated fluids. They were then given a diet low in fat (less than 2.5 mg) and low in leucine (less than 0.01 mg/kg) which they selected with the assistance of the Research Center dietician. This diet contained 60% of the total daily calories required for maintenance, served as lunch (at 4 h), dinner (at 10 h), and an evening snack (at 13 h). Each subject was served the exact same meals for all three admissions.

The metabolism of HDL was evaluated by endogenously labeling its major protein component, apoA-I, by a constant 14-h intravenous infusion of a nonradioactive isotope, trideuterated leucine (purity >99%, Tracer Technologies, Cambridge, MA), delivered at a rate of 4.8 μmol per kg body weight per h after a priming dose of 4.2 μmol per kg. This infusion maintained the plasma leucine enrichment at approximately 6% (**Fig. 1**). Blood specimens were obtained from a second intravenous catheter in the contralateral arm every 10 min for the first 90 min and hourly thereafter. Plasma was separated by centrifugation and stored at −80°C, and later underwent sequential preparative ultracentrifugation to isolate VLDL, IDL, light LDL, dense LDL, HDL_2 (d 1.063–1.125 g/ml), and HDL_3 (d 1.125–1.210 g/ml) (21). VLDL (d < 1.006 g/ml) was prepared from 4 cc plasma overlayered by 1 ml of 0.9% NaCl and centrifuged in a Beckman Type T 70.1 rotor (Beckman Instruments, Palo Alto, CA) at 70,000 rpm for 2.5 h at 7°C. A 0.4-ml portion of the infranatant underwent sequential preparative ultracentrifugation in the outer row of a Beckman Type 25 rotor at 25,000 rpm at 10°C, adjusting density with potassium bromide. Spin times of 10 h, 16 h, 20 h, 24 h, and 30 h were used to isolate IDL (d 1.006–1.019 g/ml), light LDL (d 1.019–1.035), dense LDL (d 1.035–1.063 g/ml), HDL_2 (d 1.063–1.125 g/ml), and HDL_3 (d 1.125–1.210 g/ml), respectively. Using an Abbe refractometer, densities of the specimens were confirmed by measurement of refractive index of the KBr solution in tubes centrifuged in parallel without plasma, as well as in the lipoprotein-free clear zones beneath the flotated lipoproteins. The cholesterol concentration of HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) was measured with enzymatic reagents (Boehringer-Mannheim, Indianapolis, IN) in the Lipid Research Laboratory. This laboratory is standardized for cholesterol and HDL measurements according to the program for research laboratories specified by the Centers for Disease Control and the National Heart, Lung, and Blood Institute. The apoA-I concentration of HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) was measured by radial immunodiffusion (Tago, Burlingame, CA). The standards used for measurement of apoA-I were calibrated to reference material provided by the Centers for Disease Control. The coefficients of variation for apoA-I measurements in blinded control specimens was 4.1% for HDL_2 and 5.3% for HDL_3.

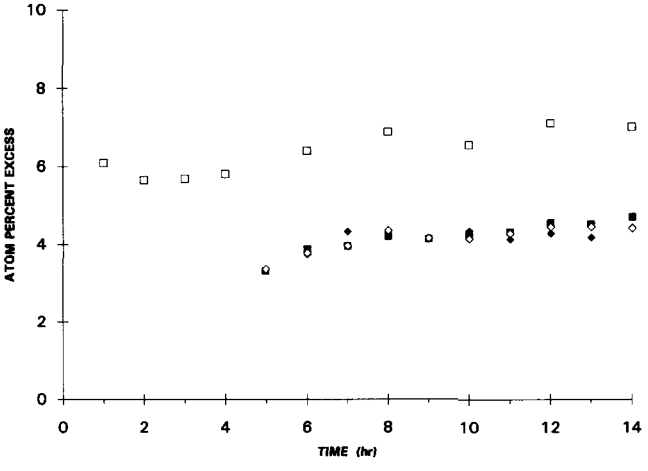

Fig. 1. Isotopic enrichment of leucine in plasma (open squares) and in VLDL apoB-100 during intravenous infusion of trideuterated leucine. Values represent mean enrichment for eight postmenopausal women during treatment with placebo (open diamonds), transdermal estradiol (closed diamonds), and oral estradiol (closed squares).

ApoA-I specimens were then prepared by SDS-polyacrylamide gel electrophoresis. HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) aliquots were combined with equal volumes of Tris SDS-BME sample buffer (Seprasol II, Enprotech, Hyde Park, MA), boiled for 5 min, and applied to precast 15% SDS-polyacrylamide gels (Enprotech). Gels were placed in a #SE600 vertical slab gel unit (Hoeffer Scientific Instruments, San Francisco, CA) with Tris-Gly-SDS tank buffer (Seprabuff, Enprotech) and run at 30 milliamp constant current for 3 h. Gels were stained (0.125% Coomassie Blue, 50% methanol, and 10% acetic acid) and destained with methanol and acetic acid. ApoA-I bands were identified by comparison with mid-range molecular weight markers (Enprotech). Bands were excised and hydrolyzed (110°C, 24 h) in 6 N HCl under nitrogen. The hydrolyzates were chilled for 20 min at −20°C and after centrifugation (3,000 rpm, 5 min, 4°C) the clear supranatant containing free amino acids was separated from precipitated polyacrylamide gel. Free leucine was extracted from hydrolyzates using AG50W-X8 resin (Bio-Rad, Richmond, CA) and eluting with 10 N NH_4OH. The specimens were completely dried by heating at 60°C under nitrogen. Amino acids were converted to volatile heptafluorobutyric acid derivatives by heating with an acetyl chloride and propanol mixture at 110°C for 25 min, then drying under N_2, and heating with heptafluorobutyric acid anhydride for 25 min at 60°C. The specimens were dried under N_2 and dissolved in ethyl acetate for injection into a 5890 gas chromatograph and a 5988A mass spectrometer (Hewlett-Packard, Palo Alto, CA). Negative chemical ionization and selective ion monitoring at m/z = 349 (for unlabeled leucine) and 352 (for labeled leucine)

were used to measure isotopic enrichment. The coefficients of variation for enrichment measurements were 4.6% for apoA-I and 3.4% for VLDL apoB. The baseline enrichment of apoA-I at time zero was 0.16%.

Calculation of metabolic rates

For each study, fractional synthetic rates (which, at steady state, are equal to fractional catabolic rates) were determined by dividing the rate of appearance of labeled leucine in HDL apoA-I by the VLDL-apoB plateau enrichment. The rate of appearance of labeled leucine in HDL apoA-I was calculated by linear regression using all the values from 2 to 14 h without "force-fitting" through the origin. The VLDL-apoB plateau enrichment was calculated by averaging the enrichments measured from 7 to 14 h (mean intraindividual coefficients of variation, 7%). This calculation is based upon the theoretical principle that the fractional synthetic rate is equal to the initial rate of appearance of isotopic label in a product (HDL apoA-I), divided by the isotopic enrichment of the precursor (intracellular leucyl-tRNA of hepatocytes and enterocytes), if the precursor enrichment is constant (22). Although the enrichment of intracellular leucyl-tRNA cannot be measured directly, it may be estimated by the plateau enrichment of a hepatic product, VLDL apoB-100, because at steady state the enrichment of a precursor and its product are equal. Although apoB-100 plateau enrichment best reflects the hepatic precursor pool enrichment, it appears to be similar to the intestinal precursor pool enrichment as well. This conclusion was based on two lines of evidence.

1) Four healthy postmenopausal women, ages 64 to 68 years, underwent a primed, 7-h continuous intravenous infusion of deuterated leucine using the same procedure as described above. They ingested 93 g of almond oil (Spectrum Naturals, Petaluma, CA) during 3 h prior to the infusion, to stimulate chylomicron production. During the infusion, they ingested 19 g of oil each hour. Phosphatidylcholine (Phoschol Concentrate, Advanced Nutritional Technology, Elizabeth, NJ), 40 mg, was added to each gram of oil to facilitate gastrointestinal absorption. No protein was given (as is our procedure for all studies) so as not to dilute out the intestinal precursor pool with unlabeled (oral) leucine. Other investigators have shown that the apoB-100 and apoB-48 enrichment curves are dissimilar when dietary leucine is administered (23). Serial blood specimens were collected and centrifuged to separate plasma. Lipoproteins with density less than 1.006 g/ml were immediately prepared from plasma overlayered with 0.9% NaCl, and spun in a Beckman Type 70.1 Ti rotor at 70,000 rpm for 2.5 h. Lipoprotein aliquots were concentrated, combined with Tris SDS-BME sample buffer (Seprasol II), boiled, and applied to 3–20% SDS-polyacrylamide gradient gels (Enprotech). Gels were run at 35 milliamp constant current for 3.5 h. ApoB-48 and apoB-100 bands were excised and processed using the same procedure as described above for apoA-I. The appearance of deuterated leucine in apoB-100 and apoB-48 for the four subjects is shown in **Fig. 2.** The isotopic enrichments of apoB-100 and apoB-48 did not differ by more than 10%, suggesting that the intestinal and hepatic precursor pools have similar isotopic enrichments. The final measurement at 7 h was 4%, which is similar to that of plateau in a 14-h infusion (Fig. 1). Thus, the VLDL-apoB-100 plateau enrichment may reflect the enrichment of both of the precursor pools for apoA-I.

2) Ikewaki et al. (24) measured apoA-I kinetics in a group of subjects with widely different apoA-I concentrations. These subjects simultaneously received a primed, continuous intravenous infusion of a stable isotope (for endogenous labeling) in conjunction with a bolus of radioiodinated apoA-I (for exogenous labeling) in order to directly compare these two methods. Using VLDL apoB-100 plateau enrichments as the estimates of apoA-I precursor pool enrichments yielded apoA-I kinetic parameters highly comparable to those obtained by the well-established radiotracer method.

Absolute production rates were calculated by multiplying fractional catabolic rates by pool sizes. Pool sizes (in mg) were calculated by multiplying plasma volumes (body weight in kilograms × 0.44) by the plasma lipoprotein concentrations (in mg per deciliter).

Statistical analysis

The treatment effect was defined as the difference in plasma lipoprotein concentrations, metabolic rates, or sex-hormone concentrations measured during the placebo period and after each estrogen treatment. The differences

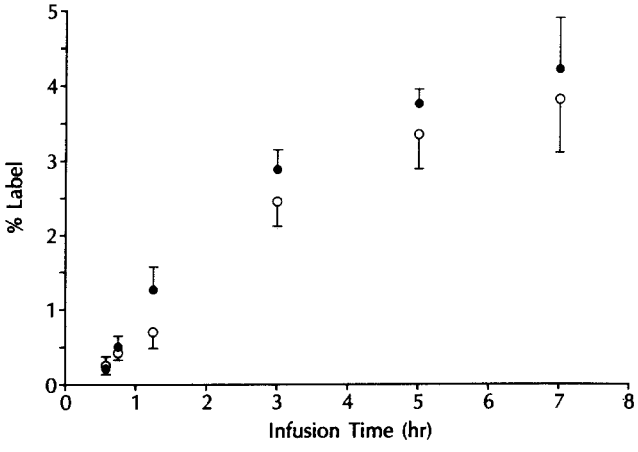

Fig. 2. Isotopic enrichment of leucine in apoB-100 (closed circles) and apoB-48 (open circles) in lipoproteins with density less than 1.006 g per milliliter, during primed continuous intravenous infusion of trideuterated leucine. Values represent mean enrichment in four healthy postmenopausal women studied during ingestion of fat. The subjects ingested 93 g almond oil over 3 h, after which the infusion of deuterated leucine was started. During the infusion, an additional 19 g of oil was given hourly. Error bars denote standard deviations.

were analyzed by analysis of variance followed by pairwise comparisons, with the Statistical Analysis System (25). Two-tailed P values were used throughout.

Comparison of preparative and analytic techniques for HDL subfractions

We have previously published (11) our results for the effect of estrogen on HDL levels subfractionated using the analytic technique of magnesium/dextran precipitation. In contrast, the present metabolic study required the use of a preparative technique to isolate HDL by sequential ultracentrifugation. As these two different methods subfractionate HDL by different physical properties, we wished to clearly define the relationships between HDL subfractions isolated by these two techniques. We therefore subfractionated HDL by sequential ultracentrifugation after subfractionation by magnesium/dextran precipitation. This was performed from the plasma obtained from three patients, during both placebo and 2 mg estradiol treatments, as follows. HDL and HDL_3 were sequentially separated by precipitation with dextran sulfate and magnesium chloride (26), and were dialyzed against phosphate-buffered saline for 90 min in an Oscillatory Microdialysis System (Bio-Tech, Bellevue, WA) using membranes with a 8000 dalton pore size. HDL and HDL_3 then underwent sequential ultracentrifugation as described above, to prepare specimens with densities less than 1.063 g/ml, 1.063–1.125 g/ml, 1.125–1.210 g/ml, and greater than 1.210 g/ml. The apoA-I content of each of these specimens was measured by immunoturbidimetry. Values for HDL_2 measured by the precipitation technique were calculated by subtraction. We found the following. *1)* There is no detectable apoA-I in specimens with density less than 1.063 g/ml. *2)* Less than 7% of total plasma apoA-I is present in specimens with density greater than 1.210 g/ml, which do not contain detectable cholesterol; this most likely represents free apoA-I unassociated with lipoprotein particles or A-I/phospholipid complexes. *3)* The apoA-I in HDL_2 determined by magnesium/dextran precipitation is present exclusively in particles with density between 1.063 and 1.125 g/ml. *4)* On average, 34% of apoA-I in HDL_3 prepared by magnesium/dextran precipitation is present in particles with density between 1.063 and 1.125 g/ml, whereas 66% is present in particles with density between 1.125 and 1.210 g/ml. Thus, HDL_2 determined by magnesium/dextran precipitation is a "narrower cut" of the HDL density spectrum, compared to HDL_2 obtained by ultracentrifugation.

RESULTS

Sex-hormone concentrations (Table 1)

FSH levels were measured to verify compliance with treatment and to compare the systemic potency of the oral and transdermal doses of estradiol. The oral and transdermal doses produced similar reductions in plasma FSH levels (34% and 41%, respectively; $P < 0.0005$ for both

TABLE 1. Plasma sex-hormone and lipoprotein concentrations during placebo, transdermal estradiol, and oral estradiol treatment

Measurement	Placebo	Estradiol	
		Transdermal	Oral
FSH, IU/liter	56 ± 8	33 ± 11**	37 ± 7**
Estradiol, pmol/liter[a]	73 ± 15	322 ± 113**	483 ± 70**
Estrone, pmol/liter[a]	172 ± 29	296 ± 55**	1954 ± 132**§§
HDL_2 (d 1.063–1.125 g/ml)			
Cholesterol, mmol/liter	1.01 ± 0.28	1.03 ± 0.20	1.42 ± 0.41*§
(mg/dl)	(39 ± 11)	(40 ± 8)	(55 ± 16)
ApoA-I, μmol/liter	27 ± 6	28 ± 5	37 ± 6**§§
(mg/dl)	(76 ± 16)	(78 ± 15)	(104 ± 18)
ApoA-I/cholesterol ratio (wt/wt)	2.0 ± 0.3	2.0 ± 0.1	2.0 ± 0.3
HDL_3 (d 1.125–1.210 g/ml)			
Cholesterol, mmol/liter	0.53 ± .07	0.49 ± 0.10	0.63 ± 0.13*
(mg/dl)	(21 ± 3)	(19 ± 4)	(24 ± 5)
ApoA-I, μmol/liter	30 ± 3	28 ± 6	33 ± 5*
(mg/dl)	(83 ± 9)	(77 ± 16)	(93 ± 14)
ApoA-I/cholesterol ratio (wt/wt)	4.0 ± 0.3	4.1 ± 0.4	3.9 ± 0.3

Values are means ± SD.
[a] To convert values for estrogens to picograms per milliliter, divide by 3.7.
*$P < 0.05$ and **$P < 0.005$, compared with the value for placebo.
§$P < 0.05$ and §§$P < 0.005$, compared with the value of transdermal estradiol.

comparisons). This degree of suppression was similar to that found previously (27) and objectively confirms compliance with treatment.

Lipoprotein concentrations

During placebo treatment, the mean concentration of total cholesterol was 187 ± 34 mg/dl (4.85 ± 0.88 mmol/liter); VLDL cholesterol, 8 ± 2 mg/dl (0.2 ± 0.06 mmol/liter); LDL cholesterol, 119 ± 32 mg/dl (3.1 ± 0.8 mmol/liter); HDL cholesterol, 60 ± 6 mg/dl (1.56 ± 0.15 mmol/liter); and total triglycerides, 68 ± 20 mg/dl (0.77 ± 0.22 mmol/liter).

HDL concentrations and metabolic rates

The major effect of oral estradiol on HDL concentrations was to increase HDL_2 (d 1.063–1.125 g/ml) cholesterol and apoA-I, by 40% ($P < 0.05$) and 37% ($P < 0.005$), respectively (Table 1). To a lesser extent, oral estradiol increased HDL_3 (d 1.125–1.210 g/ml) cholesterol and apoA-I, by 17% and 11%, respectively (P for both, < 0.05). This effect was fairly uniform with seven of eight subjects showing increases in HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) apoA-I with oral estradiol treatment (**Fig. 3A**). As oral estradiol increased cholesterol and apoA-I to the same extent, the apoA-I/cholesterol ratio was unchanged by treatment, suggesting that apoA-I may determine the cholesterol content of an HDL particle. Transdermal estradiol had no significant effect on HDL concentrations.

Both oral and transdermal estradiol had no effect on the rate of incorporation of labeled leucine into apoA-I of HDL_2 (d 1.063–1.125 g/ml) (**Fig. 4A**) and HDL_3 (d 1.125–1.210 g/ml) (Fig. 4B). Thus estradiol treatment was not found to alter the fractional catabolic rates of HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) apoA-I (Fig. 3B and **Table 2**); the 95% confidence intervals for the change in FCRs for HDL_2 (d 1.063–1.125 g/ml) was −6% to +12% and for HDL_3 (d 1.125–1.210 g/ml) was −9% to +28%. The increases in apoA-I levels produced by oral estradiol, therefore, result from an increase in apoA-I production: the rate of HDL_2 (d 1.063–1.125 g/ml) apoA-I production was increased by 36% ($P < 0.01$), and for HDL_3 (d 1.125–1.210 g/ml) apoA-I production, by 19% ($P < 0.05$), (Table 2). Seven of eight subjects showed increases in both HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) apoA-I production rates (Fig. 3C).

During each treatment, the appearance of labeled leucine into HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) was essentially simultaneous (**Fig. 5**) indicating that apoA-I rapidly cycles between HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) particles, or that rapid interconversion of these subfractions occurs.

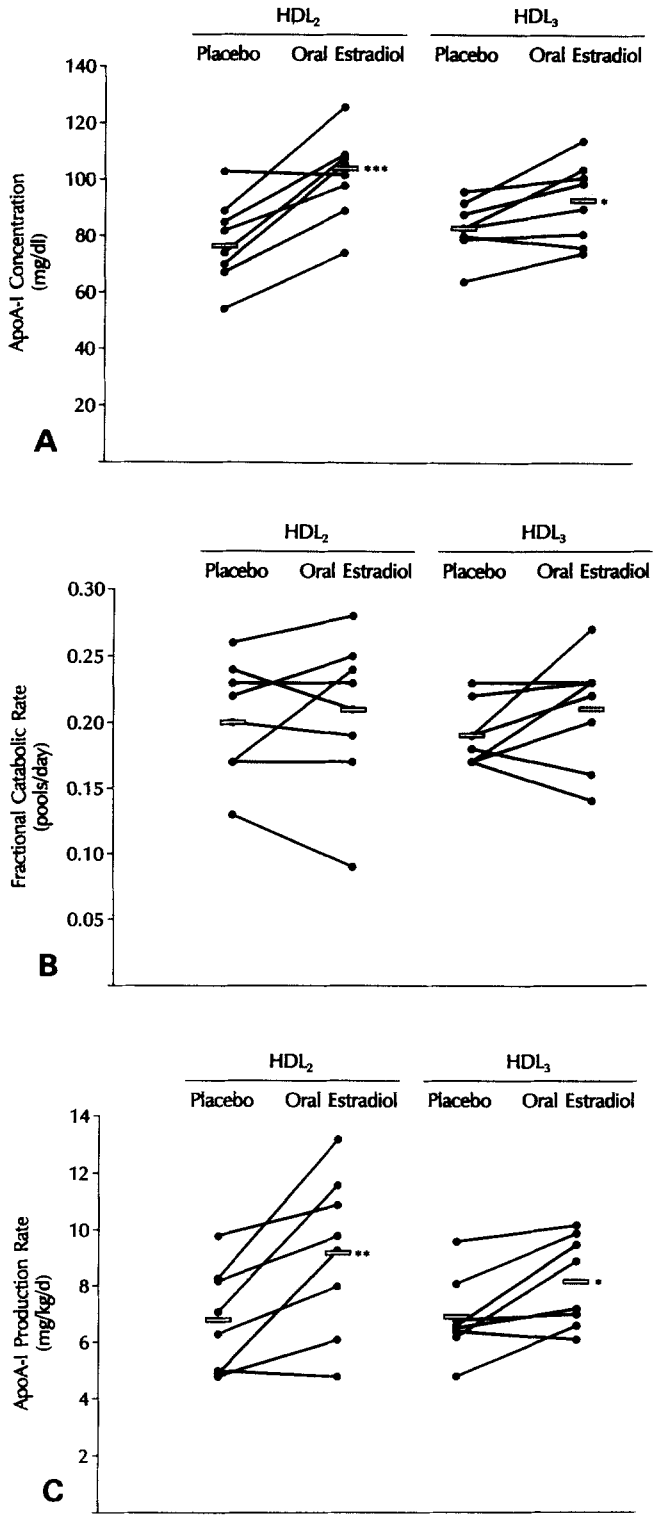

Fig. 3. ApoA-I plasma concentrations (A), fractional catabolic rates (B), and production rates (C) in HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml). Solid lines connect values for each subject (closed circles) measured during placebo and oral estradiol treatments. Mean values are represented by the horizontal bars. Asterisks denote significant changes from values during placebo treatment (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

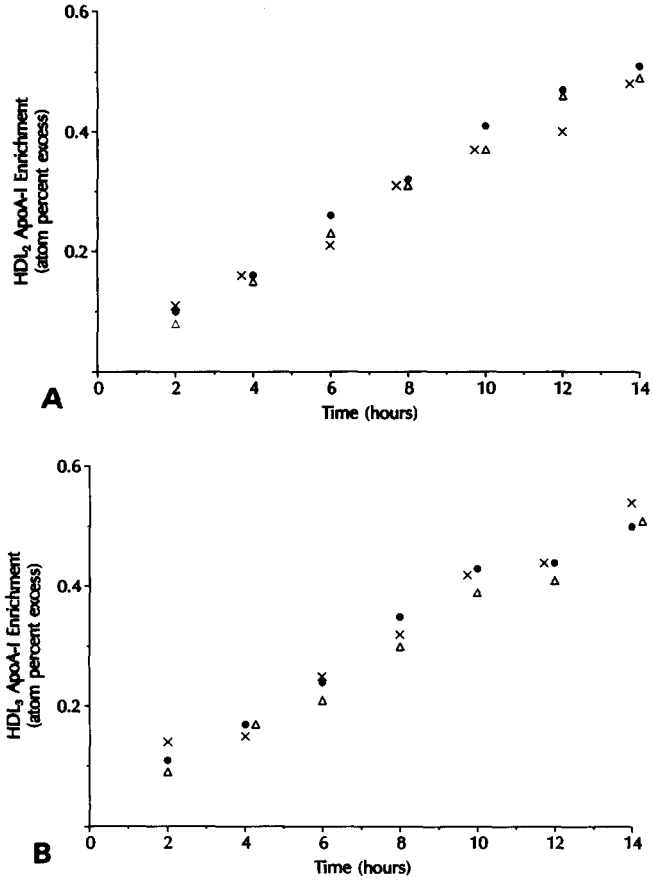

Fig. 4. Isotopic enrichment of leucine in apoA-I of HDL_2 (d 1.063–1.125 g/ml) (A) and HDL_3 (d 1.125–1.210 g/ml) (B) during intravenous infusion of trideuterated leucine. Values represent mean enrichment in all eight subjects during treatment with placebo (open triangles), transdermal estradiol (X), and oral estradiol (closed circles).

Correlations between VLDL and HDL kinetic parameters

ApoB metabolism was studied in these subjects and reported previously (11). During placebo treatment, HDL_3 (d 1.125–1.210 g/ml) apoA-I levels were highly correlated with HDL_3 (d 1.125–1.210 g/ml) apoA-I production rates ($r = 0.86$, $P = 0.005$) (**Fig. 6**), and not with HDL_3 (d 1.125–1.210 g/ml) fractional catabolic rates. HDL_3 (d 1.125–1.210 g/ml) apoA-I production rates were, in turn, highly correlated with the flux of large (S_f 60–400) VLDL-apoB ($r = 0.89$, $P = 0.002$) (**Fig. 7**). Oral estradiol treatment increased both HDL_3 (d 1.125–1.210 g/ml) apoA-I production and large VLDL-apoB production; there was a correlation between the percent increases in both parameters ($r = 0.60$, $P = 0.02$) induced by oral estradiol treatment. Similar analyses for HDL_2 (d 1.063–1.125 g/ml) apoA-I showed no such relationship.

During placebo treatment, HDL_2 (d 1.063–1.125 g/ml) apoA-I and HDL_2 (d 1.063–1.125 g/ml) cholesterol levels were correlated with the fractional catabolic rates (FCR) of large VLDL (S_f 60–400) for direct clearance from the circulation ($r = 0.73$, $P = 0.02$; and $r = 0.75$, $P = 0.02$, respectively) (**Fig. 8**). Oral estradiol treatment increased both HDL (d 1.063–1.125 g/ml) cholesterol levels and the FCR of large VLDL directly cleared from the circulation; there was a correlation between the percent changes in both parameters ($r = 0.53$, $P = 0.04$).

During all treatments, apoA-I and cholesterol levels were highly correlated in HDL_2 (d 1.063–1.125 g/ml) ($r = 0.93$, $P = 0.002$); and in HDL_3 (d 1.125–1.210 g/ml) ($r = 0.86$, $P = 0.003$).

TABLE 2. HDL apolipoprotein A metabolic parameters during placebo, transdermal estradiol, and oral estradiol treatment

Measurement	Placebo	Estradiol	
		Transdermal	Oral
	mean ± *SD*	% change from placebo value *mean* ± *SE*	
HDL_2 (d 1.063–1.125 g/ml) apoA-I			
Pool size, μmole	78 ± 19	+ 4 ± 6	+ 39 ± 6**
mg	2170 ± 530		(*P* = 0.0009)
Fractional catabolic rate, pool/d	0.20 ± 0.04	+ 8 ± 4	+ 3 ± 4
Absolute production rate, μmole/kg/d	0.24 ± 0.07	+ 7 ± 8	+ 36 ± 8*
mg/kg/d	6.8 ± 1.9		(*P* = 0.009)
HDL_3 (d 1.125–1.210 g/ml) apoA-I			
Pool size, μmole	85 ± 17	− 7 ± 5	+ 13 ± 5
mg	2380 ± 470		(*P* = 0.02)
Fractional catabolic rate, pool/d	0.19 ± 0.02	+ 14 ± 8	+ 10 ± 8
Absolute production rate, μmole/kg/d	0.25 ± 0.05	+ 4 ± 6	+ 19 ± 6
mg/kg/d	6.9 ± 1.4		(*P* = 0.04)

The general-linear-models procedure of SAS (21) was used to compute the standard error of the least-squares means of the changes.

*$P < 0.05$ and **$P < 0.005$, compared with the value for transdermal estradiol.

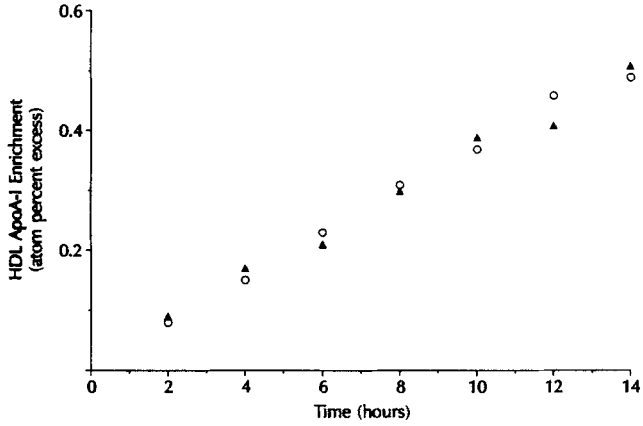

Fig. 5. Isotopic enrichment of leucine in apoA-I of HDL_2 (d 1.063–1.125 g/ml) (open circles) and of HDL_3 (d 1.125–1.210 g/ml) (closed triangles) during intravenous infusion of trideuterated leucine. Values represent means for all subjects during placebo treatment.

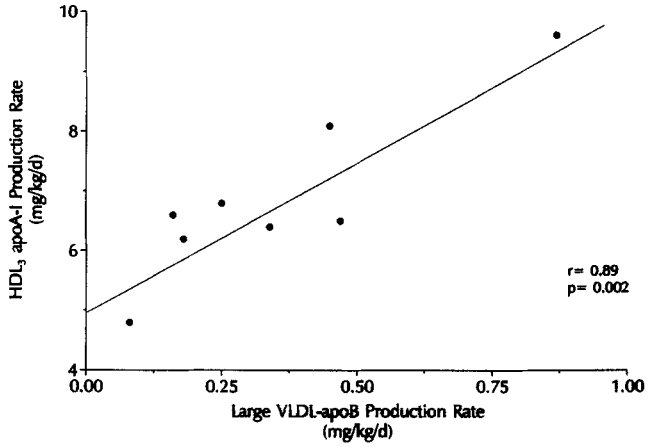

Fig. 7. Correlation analysis of HDL_3 (d 1.125–1.210 g/ml) apoA-I production rates and large (S_f 60–400) VLDL-apoB production rates during placebo treatment.

DISCUSSION

We found that a low daily dose of oral estradiol prescribed to postmenopausal women increased the plasma concentrations of HDL_2 (d 1.063–1.125 g/ml) apoA-I and HDL_3 (d 1.125–1.210 g/ml) apoA-I by 37% and 11%, respectively. This resulted entirely from increases in the rate of HDL apoA-I production and not by a reduction in the rate of apoA-I catabolism. This extends the findings of Schaefer et al. (6) who observed that a high dose of a potent synthetic estrogen, ethinyl estradiol, administered to four premenopausal women, raised HDL-protein levels by increasing the rate of HDL-protein production. Our study demonstrates that an estrogen with far less potency specifically raises apoA-I levels in both HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml) by increasing their production rates. Our findings are supported by observations in ovariectomized baboons, in whom estrogen treatment raised apoA-I levels by increasing apoA-I production (28). This effect of estrogen may be mediated by increased transcription of apoA-I mRNA, as estradiol increases secretion of both apoA-I and apoA-I mRNA in cultured human hepatoma (HepG2) cells (29). Despite the fact that estrogen has been found to decrease hepatic lipase activity (8), we found no evidence that estrogens retard the rate of HDL-apoA-I catabolism. As hepatic lipase hydrolyzes lipid ester in HDL_2 to convert it to HDL_3, a decrease in hepatic lipase activity induced by estrogen could explain the observed shift in HDL toward the HDL_2 part of the density spectrum during estrogen treatment.

We chose doses of oral and transdermal estradiol which are commonly used and clinically effective for postmenopausal estrogen replacement. The plasma estrone

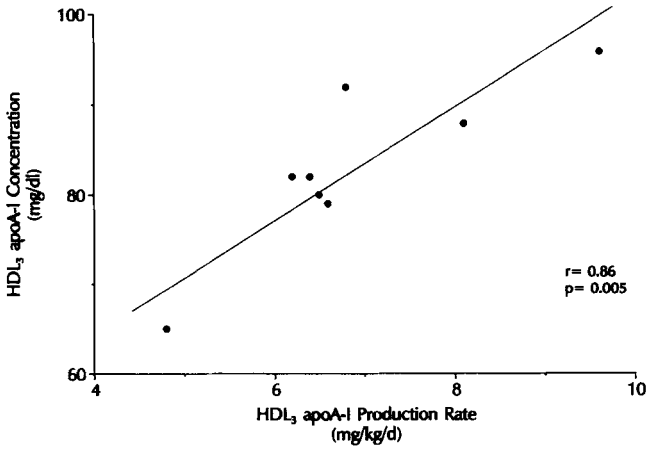

Fig. 6. Correlation analysis of HDL_3 (d 1.125–1.210 g/ml) apoA-I concentrations and the rates of HDL_3 (d 1.125–1.210 g/ml) apoA-I production, during placebo treatment.

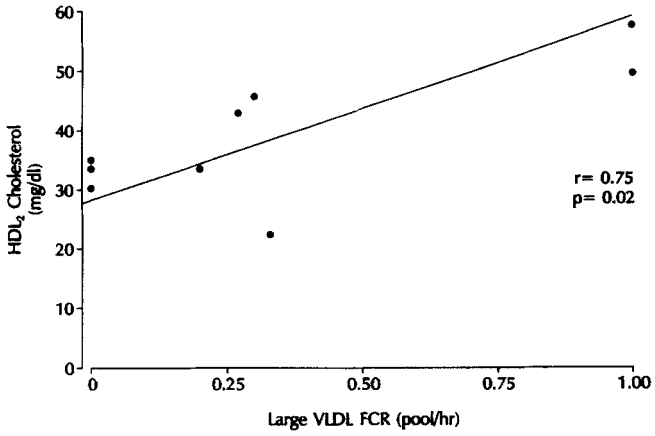

Fig. 8. Correlation analysis of HDL_2 (d 1.063–1.125 g/ml) cholesterol, and the component of the fractional catabolic rate of large VLDL (S_f 60–400) that represents direct clearance from the circulation and not conversion to small VLDL, during placebo treatment.

and estradiol levels measured during oral estradiol (near peak) and transdermal estradiol treatments agree with those previously reported (12, 30). However, they may not be a meaningful way to compare the estrogenic potencies of these two preparations as *1)* a large proportion of oral estradiol is converted to estrone, a weak estrogen; and *2)* oral estrogen enters the circulation as a bolus, which produces wide swings in plasma estrogen levels. In contrast, the transdermal application of estradiol delivers estradiol directly into the systemic circulation at a constant rate and with little conversion to estrone. Due to these differences in pharmacokinetics, it may be more meaningful to compare the estrogenic potency of these two preparations in terms of their effect on the pituitary, in suppressing FSH secretion. On that basis, both estrogens appeared to be of comparable estrogenic potency.

Although the systemic potency of both estrogens may be comparable, transdermal estradiol did not significantly alter the concentrations or metabolic rates of HDL, despite the fact that it raised plasma estradiol levels to 320 pmol per liter (88 pg per milliliter). Most (11–14) but not all (15, 16) studies in which parenteral estrogens elevated plasma estradiol concentrations to less than 440 pmol per liter (120 pg per milliliter) found minimal changes in HDL levels, whereas in studies in which estradiol levels were raised to more than 440 pmol per liter (120 pg per milliliter), HDL levels increased by 20–32% (16, 17). Thus, the effect of parenteral estrogen may be dose-dependent and evident only when plasma estradiol levels exceed those necessary for routine treatment of postmenopausal conditions. In contrast, oral estradiol with comparable systemic potency (with suppression of FSH serving as an index) did alter HDL metabolism. This effect may be due to the fact that estrogens given orally expose enterocytes and, later, hepatocytes to supraphysiologic concentrations of estrogen prior to dilution within the systemic circulation. These high intrahepatic and enteric estrogen levels may alter gut or liver metabolism so that HDL production is increased.

The metabolic rates of apoA-I that we determined by endogenous labeling are comparable to those obtained by reinfusion of radiolabeled lipoproteins in other groups of women (31, 32). The production rates of apoA-I measured in those studies were 14.8 (31) and 13.6 (32) mg/kg per day as compared with 13.7 mg/kg per day in our subjects; the fractional catabolic rates measured in those studies were 0.27 (31) and 0.24 (32) pool per day, as compared to 0.20 pool per day in our study. Indeed, the two methods appear to yield very similar values for apoA-I kinetic measurements within individuals (24). Endogenous labeling assumes that the intracellular enrichments of leucyl-tRNA of hepatocytes and enterocytes are similar. Our finding that the intestinally derived apoB-48 attained isotopic enrichment at plateau similar to hepatically derived apoB-100 suggests that this is so.

We subfractionated HDL by sequential preparative ultracentrifugation and found that HDL_2 (d 1.063–1.125 g/ml) cholesterol comprised 65% of HDL cholesterol during placebo treatment. This is comparable to the proportion (66%) of HDL_2 cholesterol observed in 129 women whose HDL was subfractionated by the same technique (33). The apoA-I/cholesterol ratio for HDL_2 (d 1.063–1.125 g/ml) (2.0) and HDL_3 (d 1.125–1.210 g/ml) (4.0) also agreed with data reported previously (34). In contrast, the proportion of HDL_2 cholesterol measured in this cohort by dextran sulfate/magnesium chloride double-precipitation was 32% (11). We found that 34% of the HDL_3 measured by precipitation floated between density 1.063–1.125 g/ml (i.e., HDL_2) in the ultracentrifuge. This difference reflects the fact that preparative ultracentrifugation separates particles solely on the basis of density, whereas polyanion precipitation separation depends on such factors as charge-charge interactions between lipoproteins and the precipitants, and is affected by pH, ionic strength, and HDL protein-lipid ratios.

We found that HDL_3 (d 1.125–1.210 g/ml) apoA-I levels during placebo treatment were determined predominantly by HDL_3 (d 1.125–1.210 g/ml) apoA-I production rates, and not by catabolic rates, in agreement with some (35, 36) but not all (37) reports. In addition, during placebo treatment, HDL_3 (d 1.125–1.210 g/ml) apoA-I production rates were highly correlated with large VLDL (S_f 60–400)-apoB production rates, and the magnitude of the increase in HDL production rates induced by estrogen was correlated with the size of the increases in VLDL-apoB production. We speculate that HDL_3 (d 1.125–1.210 g/ml) apoA-I and VLDL-apoB may share a common control mechanism for production that can be activated by estradiol. This "common control" is supported by the observations that apoA-I production rates and apoB levels are increased in tandem by high fat diets (37) and by alcohol use (38) and lowered by low fat diets (37). Alternatively, increased VLDL apoB flux may, in some way, upregulate the rate of apoA-I production.

During placebo treatment, HDL_2 (d 1.063–1.125 g/ml) apoA-I and cholesterol levels were correlated with the fractional catabolic rates of large VLDL cleared directly from the circulation. The fractional catabolic rate of large VLDL probably reflects, in large part, the activity of lipoprotein lipase in these normolipidemic subjects. This enzyme hydrolyzes the surface components of VLDL, allowing for their transfer to HDL_3, thereby converting HDL_3 to HDL_2. Prior work has demonstrated that lipoprotein lipase activity is positively correlated with HDL_2 cholesterol levels (39). Thus, increased VLDL clearance induced by estrogen would serve to convert some HDL_3 to HDL_2, thereby raising HDL_2 levels.

The enrichment curves of HDL_2 (d 1.063–1.125 g/ml) apoA-I and HDL_3 (d 1.125–1.210 g/ml) apoA-I were indistinguishable, suggesting that apoA-I exchanges freely

between HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml). This is consistent with the prior observation that when ^{125}I-labeled apoA-I/HDL_2 and ^{131}I-labeled apoA-I/HDL_3 were simultaneously injected into a subject, rapid and complete exchange of ^{125}I-labeled apoA-I and ^{131}I-labeled apoA-I occurred between the HDL_2 and HDL_3 subfractions within 10 min (40). Thus, differences in the metabolic rates of HDL_2 and HDL_3 particles cannot be detected using apoA-I as a marker.

We further noted that estrogen treatment did not alter the fractional catabolic rates of HDL, despite the fact that plasma triglyceride levels were increased by 24%. Although other investigators (32) have suggested that elevations in triglyceride levels increase the fractional catabolic rates of HDL, our data as well as that of others (41) indicate that this is not always the case. It may be that the relationship between high triglyceride levels and high HDL FCRs found in hypertriglyceridemics does not apply among estrogen users, where increased triglyceride levels instead occur due to increased VLDL production and not by impaired VLDL catabolism (11). This effect of estrogen appears to simular to that of alcohol.

The increases in HDL levels caused by estrogen may, if sustained over many years, protect against the development of cardiovascular disease. HDL has been reported to bind to macrophages to stimulate the efflux of cholesterol (42) as well as to retard the oxidation of LDL (43). Moreover, insertion of an apoA-I gene into transgenic mice has been found to prevent diet-induced atherogenesis (44). Based on the experience of two clinical trials in which HDL cholesterol levels were raised by drug treatment (45, 46), estrogen-induced increases in HDL may decrease the risk of cardiovascular disease by as much as 40%.

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