Sequential Estrogen-Progestin Replacement Therapy in Healthy Postmenopausal Women: Effects on Cholesterol Efflux Capacity and Key Proteins Regulating High-Density Lipoprotein Levels

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Thirty healthy postmenopausal women were randomized into 2 groups that received a sequential combined hormone-replacement therapy (HRT) (n = 18; conjugated equine estrogen 0.625 mg/d for 28 days and 5 mg of medroxyprogesterone acetate during the last 14 days) or placebo (n = 12). Plasma samples were collected before and during treatment (days 0, 15, 43, 71). High-density lipoprotein (HDL) lipid content, lipoprotein (Lp)A-I and LpA-I:LpA-II concentration, lecithin:cholesterol acyl transferase activity (LCAT), phospholipid transfer protein (PLTP) activity, and the plasma capacity to carry out cholesterol efflux from Fu5AH cells were measured. Most significant changes were found within the first 15 days after HRT. After 71 days of HRT, we found an increase in LpA-I lipoparticles (27%) and the following HDL lipids: phospholipids (21%), triglycerides (45%), and free cholesterol (43%), as well as an increase in cholesterol efflux (12.5%). PLTP activity, on the other hand, decreased 21% after 71 days of treatment. No significant changes in LCAT activity, HDL-cholesterol ester or LpA-I:LpA-II particles were found. Positive correlation between cholesterol efflux and the variables LpA-I and HDL-phospholipids were observed. PLTP was negatively correlated with apolipoprotein (apo) A-I, LpA-I, and LpA-I:LpA-II. In summary, our study, performed during 3 hormonal cycles, shows that HRT not only modifies HDL-cholesterol level, but also its lipid composition and HDL lipoparticle distribution. HRT enhances the plasma capacity to carry out cholesterol efflux from the Fu5AH system and decreases the activity of PLTP, a key protein regulating HDL levels. Considering the protocol sampling, these results represent mainly the estrogenic effect of HRT.

CARDIOVASCULAR DISEASE is the major cause of death for women in the Western world. Approximately 500,000 women die of this disorder each year in the United States.[1] In Chile, death from circulatory disease also constitutes the leading cause of mortality for women.[2]

There is considerable epidemiologic evidence, mainly observational studies, suggesting that estrogen deficiency in postmenopausal women increases their risk of cardiovascular disease, particularly coronary heart disease (CHD), and that postmenopausal estrogen therapy can reduce the increased risk.[3] Current evidence reveals that 25% to 50% of the beneficial effects related to estrogen therapy can be attributed to its influence on plasma lipoprotein levels. Moreover, there are several other beneficial effects of estrogens on cardiovascular risk factors that could also explain why it is advantageous.[4] At the same time, however, estrogen therapy may have potentially detrimental effects on cardiovascular biomarkers, such as increasing triglyceride levels and activating coagulation.[5] The Heart and Estrogen-Progestin Replacement Study (HERS) and other randomized trials have not demonstrated benefits in secondary prevention among women with pre-existing CHD.[6,7] HERS investigators suggest that estrogen may cause thrombosis, arrhythmia, or ischemia. Nevertheless, it has been reported that morbidity/mortality from CHD in women in the United States has declined substantially in the past 3 decades,[8] and the increase in the use of hormone-replacement therapy (HRT) during menopause appears to be, in part, responsible for this effect.[9,10]

There is consensus that the cardioprotective effect of HRT is to some extent secondary to changes in the plasma lipid profile, mainly the increase in high-density lipoprotein (HDL) cholesterol. Since this therapy constitutes a potent method for raising HDL levels[11] it may be useful for exploring the mechanisms that regulate HDL metabolism.

HDL plasma level is a strong inverse predictor of CHD.[12] This lipoprotein is considered to be antiatherogenic due to its key role in the reverse cholesterol transport (RCT), a pathway by which excess cholesterol is removed from the peripheral cells and transported to the liver for elimination in bile.[13] The first step of RCT begins with cholesterol efflux from cell membranes to pre-β_1-HDL, which are the first acceptors of cell-derived free cholesterol.[14] Thereafter, lecithin:cholesterol acyl transferase (LCAT) catalyzes the cholesterol esterification in these particles,[15] and HDL-cholesterol ester can be transferred by cholesteryl-ester transfer protein (CETP) to triglyceride-rich lipoproteins (very–low-density lipoprotein [VLDL] or low-density lipoprotein [LDL]).[16] It is important to point out that the transference mediated by CETP can be antiatherogenic because it increases the rate of the RCT; however, the fact that CETP redistributes cholesterol-esters from the antiatherogenic HDL to the potencially atherogenic VLDL/LDL implies that it may also be proatherogenic.[17] Nevertheless, cholesterol esters from HDL can bypass CETP and be directly taken up by the

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liver through the scavenger receptor class B-I (SRBI) pathway.[18]

In addition to CETP, the plasma contains another lipid transfer protein known as phospholipid transfer protein (PLTP), which has been cloned.[19] It has been proposed that PLTP might exert a dual beneficial effect on RCT through the concomitant generation of pre-β-HDLs, the initial acceptors of cellular cholesterol, and large cholesteryl ester–rich α-HDLs, which can carry large amounts of cholesterol back to the liver.[20] Furthermore, PLTP transports postlipolytic surface remnants, mainly phospholipids, to the HDL fraction, and via this function participates in the maintenance of plasma HDL levels.[21] In other words, PLTP might participate in the first and last step of RCT.

HDL is a heterogeneous entity containing 2 main lipoparticle subpopulations that can be isolated by immunoaffinity chromatography. These subfractions are identified as those containing both apolipoproteins (apo) A-I and A-II, called lipoprotein (Lp)A-I:A-II, and those that only contain apo A-I, called LpA-I.[22] Since premenopausal women have higher levels of LpA-I than age-matched men,[23] it is possible to speculate that estrogen could selectively increase these lipoparticle levels. In vitro studies have concluded that the apo A-I and apo A-II content of HDL is one determinant in its ability to promote cholesterol efflux from cell membranes.[24] In addition, a higher association between cholesterol efflux and plasma LpA-I than LpA-I:A-II was demonstrated, suggesting that LpA-I is a better cholesterol acceptor from cell membranes.[25]

The aim of this study was to evaluate the effect of sequential combined estrogen-progestin replacement therapy at the unopposed estrogenic phase, on the lipid composition of HDL, the concentration of LpA-I and LpA-I:A-II particles, the plasma capacity to carry out cholesterol efflux from Fu5AH cells, and the activities of PLTP and LCAT (2 key proteins regulating HDL levels), in a group of healthy postmenopausal women.

MATERIALS AND METHODS

Thirty healthy Chilean postmenopausal women were recruited from the Menopause Clinic, Pontificia Universidad Católica, Chile. A randomized, double-blind experiment was conducted. The women included in the study were those with amenorrhea for a minimum of 1 year and presenting typical climacteric symptoms, plasma follicle-stimulating hormone (FSH) levels greater than 50 IU/L, and moderate dyslipidemia. These women also had a normal mammography and Papanicolaou smear, and none had contraindications for hormonal substitution. The exclusion criteria were tobacco consumption, ethanol intake of more than 28 g/d, diabetes, thyroid or hepatic disease, and use of drugs affecting lipid metabolism or blood pressure. Informed consent was obtained from all participants, according to the guidelines of the Declaration of Helsinski, and the protocol was approved by the Research Commission of the School of Medicine, Pontificia Universidad Católica, Chile.

Women were randomized into 2 groups considering a ratio of 1.5/1 between treated and placebo groups, respectively. The first group (n = 18) was treated with 3 cycles of sequential combined oral HRT, consisting of 0.625 mg conjugated equine estrogen for 28 days and 5 mg of medroxiprogesterone acetate (progestin) during the last 14 days of each cycle. The other group (n = 12) received a placebo. Patients were instructed to maintain a stable diet during the study. Two women dropped out from the study, one in each group, due to personal reasons. As shown in Table 1, both groups were similar in terms of age, body weight, and body mass index. Physical examination and evaluation of protocol compliance were performed before and after 71 days of therapy. Venous blood samples using EDTA (1 mg/mL) were obtained after overnight fasting, and before (0 days) and after 15, 43, and 71 days of therapy, corresponding to the last day of estrogen administration, just before the addition of progestin in each cycle. Plasma samples were immediately obtained by centrifugation, aliquoted and stored at -80°C.

Table 1. Clinical Characteristics of HRT and Placebo Groups

	HRT (n = 17)	Placebo (n = 11)	*P*
Age (yr)	53.8 ± 1.0	55.1 ± 1.2	NS
Weight (kg)	65.0 ± 2.2	68.3 ± 3.0	NS
BMI (kg/m^2)	28.6 ± 1.1	29.9 ± 1.3	NS

NOTE. Values are mean ± SE.
Abbreviations: BMI, body mass index; NS, not significant.

Analytical Determinations

Total lipids and apolipoproteins. Plasma cholesterol, triglycerides, and phospholipids were measured by enzymatic methods. HDL lipids (total cholesterol, free cholesterol, phospholipids, and triglycerides) were determined in the floating fraction after precipitation of apo B–containing lipoproteins with sodium phosphotungstic in the presence of magnesium chloride. Cholesterol ester from HDL was calculated from the difference between total and free cholesterol for this lipoprotein. LDL-cholesterol was calculated using the Fridewald's formula. HDL–free cholesterol was determined using a kit from Boehringer-Mannheim (Mannheim, [4]Germany); other lipids were determined using reactive kits from Bio-Mèrieux (Lyon, France). Apolipoproteins A-I and B were measured by immunoturbidimetric methods using kits from Roche (Basel, Switzerland).

Particles LpA-I and LpA-I:A-II. Quantification of LpA-I and LpA-I:A-II particles was performed using a kit from Sebia (Issy les Molineaux, France). In addition, LpA-I particles were measured by sandwich enzyme-linked immunosorbent assay (ELISA) as previously described[26] using monoclonal antibodies produced and characterized in our laboratory.[27,28]

Cholesterol efflux assay. The efflux capacity of each plasma sample was determined using hepatoma rat cells, Fu5AH, as previously described.[25] This assay measures the potential of the plasma specimen to remove radiolabeled cholesterol from the cell membranes. Briefly, cell monolayers were obtained after 48 hours in modified Eagle's medium (MEM) containing 5% fetal calf serum (FCS). Thereafter, 25×10^3 cells were added to 12-well plates. After 2 days, Fu5AH cells were labeled with ^{3}H-cholesterol during 60 hours (1 μCi/well). In order to obtain equilibrium for labeling, cells were washed and incubated for 24 hours in MEM containing 0.5% bovine serum albumin (BSA). For the cholesterol efflux assay, cells were extensively washed with phosphate-buffered saline (PBS) and then incubated with a 2.5% dilution of patient's plasma for 3 hours at 37°C. Then, the incubation medium was removed, and the cell monolayer washed with PBS and also removed using 0.5 mL of 0.1 mol/L NaOH. Radioactivity was measured in both incubation medium and cells. The radioactivity released to the medium was expressed as the fraction of the total radioactivity present in the well. Negative controls (blank without plasma) and positive controls (HDL_3) were run in each assay and all determinations were performed in triplicate.

LCAT activity. LCAT activity (EC 2.3.1.43) was determined using fresh discoidal artificial liposomes as a substrate. Liposomes containing phosphatidylcholine (PC) (2.49 μmol/mL), cholesterol (0.125 μmol/mL), ^{3}H-cholesterol (10^6 cpm), and apo A-I (0.010 μmol/mL) were prepared by the sodium cholate dispersion method described by Matz

Table 2. Lipid Profile From Treated (HRT) and Placebo Groups

	T_0	T_{15}	T_{43}	T_{71}	% Variation T_{71} *v* T_0
HRT (n = 17)					
Total cholesterol	211 ± 9.6	190 ± 6.7*	187 ± 8.4*	193 ± 8.1*	−8.5†
LDL-cholesterol	147 ± 8.1	115 ± 6.5*	111 ± 8.0*	117 ± 7.9*	−20.4†
Total triglycerides	113 ± 11.7	146 ± 12.4*	149 ± 15.2*	152 ± 13.0*	+34.5
HDL-cholesterol	41 ± 1.6	46 ± 1.8*	46 ± 2.2*	46 ± 1.9*	+12.2†
Total phospholipids	209 ± 10.2	219 ± 8.9	227 ± 10.7*	231 ± 10.7*	+10.5†
Apo A-I	134 ± 2.7	142 ± 2.8*	151 ± 3.5*	150 ± 3.2*	+11.9†
Apo B	104 ± 6.9	93 ± 6.6*	89 ± 5.9*	90 ± 5.6*	−13.5†
Placebo (n = 11)					
Total cholesterol	221 ± 13.7	227 ± 12.3	221 ± 9.8	219 ± 10.7	−0.9
LDL-cholesterol	155 ± 13.5	159 ± 11.6	158 ± 7.8	152 ± 8.8	−1.9
Total triglycerides	127 ± 21.4	154 ± 21.1	131 ± 16.8	144 ± 20.1	+13.3
HDL-cholesterol	40 ± 2.4	38 ± 2.4	38 ± 2.3	38 ± 1.9	−5
Total phospholipids	223 ± 14.4	216 ± 9.4	221 ± 10.2	213 ± 10.0	−4.5
Apo A-I	140 ± 4.2	137 ± 4.9	140 ± 3.6	138 ± 3.6	−1.4
Apo B	118 ± 9.6	119 ± 7.6	117 ± 7.9	119 ± 7.6	+0.8

NOTE. Results are presented as the mean ± SE (mg/dL) at different times of treatment (T_0, basal; T_{15}, 15 days; T_{43}, 43 days; T_{71}, 71 days of therapy).

*$P < .05$ in comparison to baseline.

†$P < .05$ in comparison to placebo group.

and Jonas.[29] LCAT activity was determined as described by Ulloa et al[30] with some modifications. Briefly, 25 μL plasma was preincubated for 5 minutes at 37°C with 20 μL β-mercaptoethanol (100 mmol/L), 10 μL human albumin free fatty acid (50 μg/mL), and 70 μL Tris buffer (Tris 100 mmol/L, NaCl 0.14 mol/L, and EDTA 1 mmol/L, pH 7.4). The reaction was started by adding 100 μL liposomes to the plasma, which was then incubated at 37°C for 25 minutes in a shaking bath. The reaction was stopped by the addition of 0.5 mL methanol. Lipids were extracted from the reaction mixture with 1 mL n-hexane and vortexed for 30 seconds at standard speed. Finally, 600 μL of the extract was used for column chromatography with activated silica gel G (0.5 g) and esterified cholesterol was selectively eluted with 3 mL diethyl ether:n-hexane 6:1 (vol/vol). Radioactivity of this eluate was measured in a scintillation counter (Packard Instruments, Meriden, CT). Negative controls (blank without plasma) were run in each assay and all determinations were performed in triplicate.

Assay of PLTP activity. The assay was carried out as described by Jauhiainen et al with some modifications.[31] Liposomes containing 10 μmol of egg PC, 1 μCi of ^{14}C-dipalmitoyl-PC, and 20 nmol of butylated hydroxytoluene were prepared as previously described.[32] Each assay contained HDL_3 as acceptor (250 μg of protein), donor liposomes (150 nmol of ^{14}C-PC–labeled liposomes), sample (4 μL of 1:10 diluted human plasma), and buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) in a final volume of 400 μL. Determinations were run in duplicate. Tubes without samples and tubes with a control plasma sample were included in each run. The assay was performed at 37°C in Eppendorf tubes and the reaction mixtures were incubated for 90 minutes. The reaction was stopped by addition of 0.3 mL of a stop-mixture (536 mmol/L NaCl , 363 mmol/L $MnCl_2$, 52 U of heparin), yielding a final concentration of 230 mmol/L NaCl, 156 mmol/L $MnCl_2$ and 74 U of heparin per milliliter. The tubes were vortexed for 10 seconds, left to stand for 10 minutes at room temperature, and then centrifuged for 10 minutes at 15,000 rpm. Supernatants (0.5 mL) were used for radioactivity determinations. The results are expressed as nanomoles of PC transferred from PC liposomes to HDL.

Statistical Analysis

Results are expressed as the mean ± SE. Intergroup comparisons, treated versus placebo values, were performed by nonparametric Mann-Whitney test. Intragroup variations, before versus after therapy, were assessed by Wilcoxon signed-rank test. Pearson correlation coefficients were determined. Data were processed using GraphPad Prism Software (San Diego, CA). Regarding the cholesterol efflux assays, a global analysis between and within groups was performed using mixed effect models (also known as analysis of variance [ANOVA] for repeated measures). In this context, ANOVA-type F statistics were used to compare changes overtime and differences between the 2 groups. To reduce the between-subject variability when comparing the 2 groups over time, baseline values were used as a covariate in the model. To confirm this analysis, changes from baseline were compared between the 2 groups. The SAS/STAT program package (SAS Insitute, Cary, NC) was used for the mixed effect model.

RESULTS

Lipid Profile

HRT after 71 days produced a significant increase in HDL-cholesterol (+12.2%, $P = .002$), triglycerides (+34.5%, $P = .026$), total phospholipids (+10.5%, $P = .015$), and apo A-I (+11.9%, $P = .027$), and a significant decrease in total cholesterol (-8.5%, $P = .033$), LDL-cholesterol (-20.4%, $P = .001$), and apo B (-13.5, $P = .0006$) . These changes occurred early on and reached statistical significance in comparison to baseline after 15 days of treatment. In contrast, the placebo group did not show significant differences for any of these parameters. All lipids and apolipoproteins after 71 days of treatment in the HRT group reached values significantly different in comparison to the placebo group ($P < .05$) (Table 2).

Evaluation of HDL Lipid Composition

The HDL lipid composition consisting of HDL–cholesterol ester, HDL–free cholesterol, HDL-phospholipids, and HDL-triglycerides showed significant variation during HRT (Table 3).

As shown in Table 2, the HDL content of cholesterol increased rapidly at 15 days of therapy and reached a mean

Table 3. HDL Lipid Content From Treated (HRT) and Placebo Groups

	T_0	T_{15}	T_{43}	T_{71}	% Variation T_{71} *v* T_0
HRT (n = 17)					
Free cholesterol	10.3 ± 0.6	13.3 ± 0.7*	15.2 ± 1.9*	14.7 ± 0.7*	+42.6†
Cholesterol ester	31.7 ± 1.8	32.8 ± 1.6	30.7 ± 2.1	31.2 ± 1.6	−1.6
Phospholipids	70.9 ± 2.1	82.1 ± 2.1*	83.1 ± 2.9*	85.8 ± 2.5*	+21.0†
Triglycerides	13.2 ± 1.1	19.0 ± 2.2*	17.6 ± 1.9*	19.1 ± 1.9*	+44.6†
Placebo (n = 11)					
Free cholesterol	9.9 ± 1.0	9.2 ± 0.9	8.6 ± 0.8	9.2 ± 0.8	−7.1
Cholesterol ester	29.8 ± 1.7	28.3 ± 1.7	28.9 ± 1.8	28.9 ± 1.3	−3.0
Phospholipids	72.9 ± 3.6	72.4 ± 3.3	68.5 ± 4.1	71.7 ± 4.1	−1.6
Triglycerides	13.7 ± 2.0	15.8 ± 2.9	11.2 ± 0.7	14.3 ± 2.2	+4.3

NOTE. Results are presented as mean ± SE (mg/dL) at different times of treatment (T_0, basal; T_{15}, 15 days; T_{43}, 43 days; T_{71}, 71 days of therapy).
*$P < .05$ in comparison to baseline.
†$P < .05$ in comparison to placebo group.

increase of 12.2% above baseline at day 71 ($P = .002$). This increment was due almost exclusively to the increase of HDL–free cholesterol, which reached a level that was 42.6% above the baseline at day 71 of HRT ($P = .0001$). The cholesterol ester of HDL remained unchanged. Phospholipids and triglycerides of HDL also showed a significant increase of 21% ($P = .0003$) and 44.6% ($P = .0212$), respectively, after 71 days of HRT in comparison to baseline. No significant differences were observed in HDL lipids in the placebo group.

Evaluation of LpA-I and LpA-I:A-II Particles

HRT produced an early increase in LpA-I particles at 15 days post-therapy with a mean increment of 27% over the basal value ($P = .0002$), and this effect was maintained during the evaluation period (Fig 1A). No significant differences were observed in the placebo group. The intergroup comparison revealed a significantly higher value in the HRT group than in the placebo group ($P = .0034$) (Fig 1A). There was not a significant difference in the LpA-I:A-II concentration in either the HRT or placebo group (Fig 1B).

Cholesterol Efflux Evaluation in Fu5AH Cells

The basal values of cholesterol efflux did not display significant differences between HRT (mean, 18.73 ± 1.17) and placebo (mean, 19.86 ± 1.87). As shown in Fig 2, there was a significant difference in the response of the cholesterol efflux between the 2 groups starting at day 15 of HRT, which remained constant through the entire period ($P < .001$). While the HRT group significantly increased the cholesterol efflux by 12.5% over the baseline value at day 71, the placebo group did not show significant modifications during treatment.

LCAT Activity

LCAT activity was not modified in either group. The mean LCAT activity at baseline was 132 ± 10.0 nmol/h/mL for the

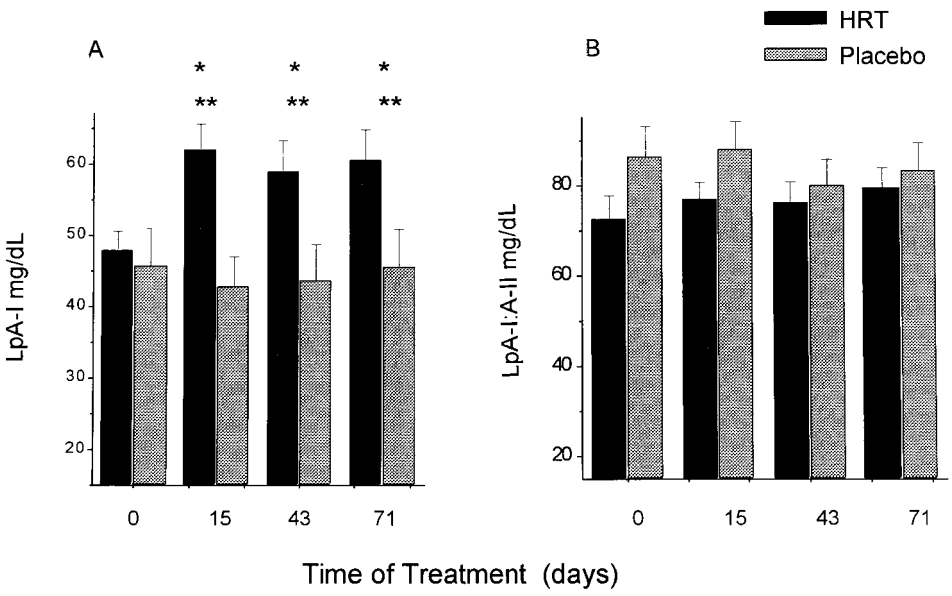

Fig 1. (A) LpA-I and (B) LpA-I:A-II concentrations during HRT or placebo treatment. Values are mean ± SE. *Significantly different from baseline ($P < .001$). **Significantly different as compared with placebo group ($P < .05$).

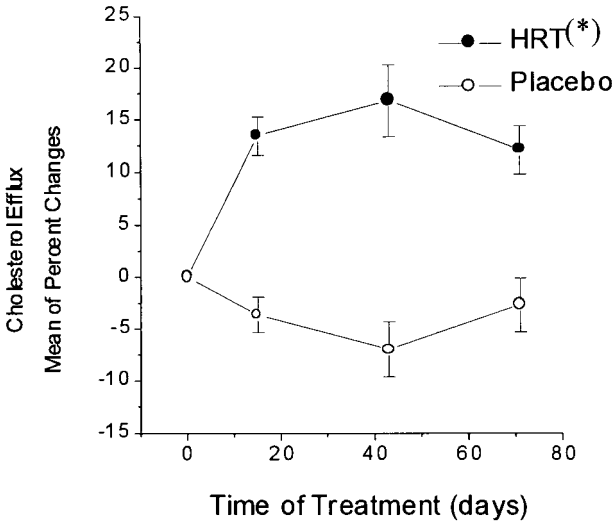

Fig 2. Cholesterol efflux in the HRT and placebo groups. Data are expressed as mean of percent changes calculated from the difference between cholesterol efflux through treatment and the basal value for each woman included in HRT or placebo groups. Vertical lines indicate SEM. *Significantly different as compared with placebo group ($P < .001$).

HRT group and 139 ± 7.2 nmol/h/mL for the placebo group. No significant changes were seen during HRT: 142 ± 8.5 nmol/h/mL, 150 ± 25.0 nmol/h/mL, and 133 ± 7.7 nmol/h/mL after 15, 43, and 71 days post-therapy, respectively (P values are not significant with respect to the baseline or placebo group; data not shown).

PLTP Activity

As shown in Fig 3, PLTP activity was significantly decreased after 71 days post-therapy to a mean of 21% under the baseline ($P = .0088$), and this modification was also significant when compared with the placebo group ($P = .0103$). No significant changes were observed early on, at 15 or 43 days post-HRT.

Furthermore, we analyzed the relationship between PLTP activity and all of the parameters measured. We found negative correlations between PLTP activity and the following: LpA-I: A-II ($r = -0.37$, $P < .0001$), LpA-I ($r = -0.29$, $P = .002$), and apo A-I ($r = -0.20$, $P = .03$).

Correlation of HDL Structural Component and Cholesterol Efflux

We analyzed the relationship between HDL structure (lipoparticle or lipid contents) and function (cholesterol efflux) in all of the samples from both groups (n = 112). Positive correlations were found between cholesterol efflux and LpA-I lipoparticles ($r = 0.32$, $P = .0004$; Fig 4A), HDL-phospholipids ($r = 0.30$, $P = .0012$; Fig 4B), HDL–free cholesterol ($r = 0.22$, $P = .018$), and apo A-I ($r = 0.30$, $P = .001$). The other HDL lipids did not demonstrate significant correlations with cholesterol efflux.

On the other hand, statistical analyses showed that several HDL structural components are positively correlated between themselves. HDL-phospholipids/LpA-I ($r = 0.65$, $P < .0001$), HDL-phospholipids/apo A-I ($r = 0.52$, $P < .0001$), HDL-phospholipids/free cholesterol ($r = 0.63$, $P < .0001$), LpA-I/apo A-I($r = 0.55$, $P < .0001$), LpA-I/free cholesterol ($r = 0.37$, $P < .0001$), and apo A-I/HDL–free cholesterol ($r = 0.31$, $P = .0009$). This statistical behavior leads us to speculate that perhaps there exists an interdependence between apo A-I concentration and free cholesterol and phospholipid levels contained in HDLs.

DISCUSSION

The present study shows that short-term estrogen-progestin HRT in healthy postmenopausal women produces changes in the lipid content of the HDL fraction, selectively increases LpA-I subpopulation, decreases PLTP activity, and enhances the plasma cholesterol efflux capacity. In addition, HRT produces beneficial changes in the lipid profile (ie, decrease in total cholesterol, LDL-cholesterol, and apo B; and increase in HDL-cholesterol and apo A-I), but also increases total triglycerides. These results are in agreement with other reports.[33,34]

Besides the well-known positive effect of HRT on HDL-cholesterol, our data provide additional information concerning the lipid composition and the subpopulation distribution of this lipoprotein. The percentage of different HDL lipids increased significantly during HRT. The mean percentage increase was 44.6% for triglycerides, 42.7% for free cholesterol, and 21% for phospholipids. The level of cholesterol ester remained unchanged.

On the other hand, the HDL subpopulation containing only apo A-I (LpA-I) was selectively increased by HRT, reaching a level of 27% above the baseline. Another study supporting our finding is that of Brinton, who demonstrated that oral estrogen replacement therapy in 6 surgically postmenopausal women selectively raised LpA-I levels.[35] The increase in LpA-I as a

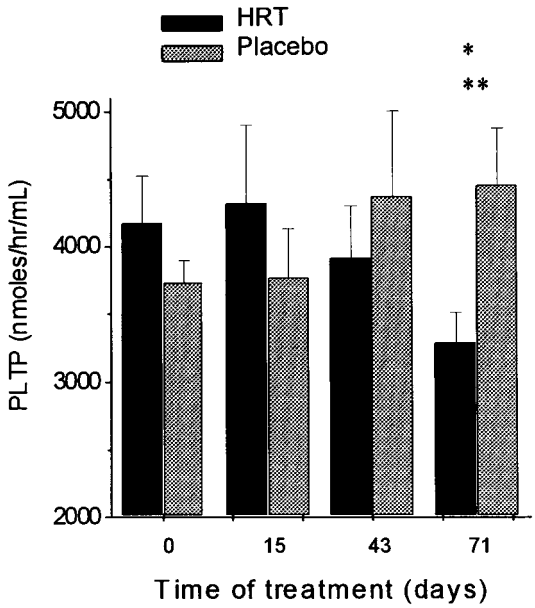

Fig 3. PLTP activity during HRT or placebo treatment. Values are mean ± SE. *Significantly different from baseline ($P < .005$). **Significantly different as compared with placebo group ($P < .01$).

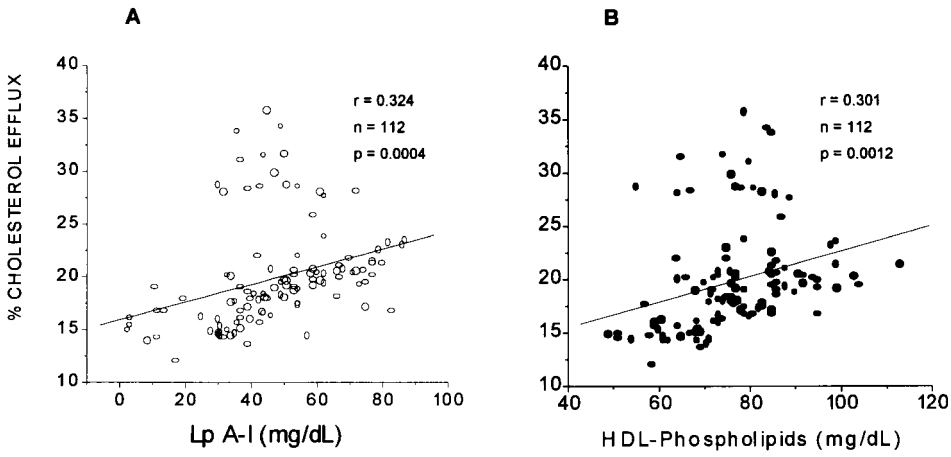

Fig 4. (A) Correlations between LpA-I levels and percentage of cholesterol efflux from Fu5AH cells carried out by plasma samples (HRT and placebo groups). (B) Relationship between HDL-phospholipid levels and percentage of cholesterol efflux from Fu5AH cells carried out by plasma samples (HRT and placebo groups).

consequence of HRT is in agreement with previous in vitro findings using HepG2 cells, where it was demonstrated that estradiol stimulates a selective increase in ^{3}H-Leu incorporation into LpA-I but not LpA-I:A-II particles and also stimulates transcription of hepatic apo A-I mRNA without affecting its half-life.[36] Since LpA-I appears to be a better cholesterol acceptor in some in vitro systems,[24,25] increased levels could explain to a certain extent the antiatherogenic effect of HRT. However, epidemiologic insights concerning LpA-I and LpA-I:A-II lipoparticles as protective HDL subfractions are still controversial; therefore, it cannot be established that LpA-I is the only antiatherogenic subpopulation. Some cross-sectional epidemiologic studies have suggested that LpA-I is apparently more antiatherogenic than LpA-I:A-II.[37-39] In addition, a case-control study showed a lower LpA-I level associated to higher incidence of CHD in Northern Ireland than France, where the incidence of CHD is 3-fold lower.[40] However, a recent prospective study considering environmental factors (Prospective Epidemiological Study of Myocardial Infarction or PRIME) showed that LpA-I and LpA-I:A-II were both reduced in male survivors of myocardial infarction from Northem Ireland or France, suggesting that a decrease in both particles is a risk marker of CHD.[41] Therefore, the question if LpA-I is or is not more protective than LpA-I:A-II still remains unanswered.

The most important finding in our study was that HRT significantly increased the plasma cholesterol efflux capacity as determined using Fu5AH rat hepatoma cells in conditions previously validated.[25] The increase in cholesterol efflux reached a mean value that was 12.5% over baseline. This value is very close to the mean increase of HDL-cholesterol (12.4%) during HRT, a result that agrees with the correlation found between HDL and cholesterol efflux.[25] The mechanism by which cholesterol efflux takes place in the Fu5AH system appears to be mediated by an interaction of extracellular acceptors with the SRBI receptor, which is highly expressed in this cell type,[42] but we cannot exclude that a passive efflux could be occurring.

The positive relationship between LpA-I lipoparticles and cholesterol efflux determined in vitro using the Fu5AH system (Fig 4A) suggests that HRT may optimize the cholesterol efflux in vivo. The positive relationship between cholesterol efflux and phospholipid from HDL (Fig 4B) agrees with previous in vitro studies reporting a strong positive correlation between both variables.[43] It has been postulated that the cholesterol efflux from plasma membranes to HDL could be accomplished by 2 mechanisms. First, if HDL is rich in lipids (α-HDL), it is believed that free cholesterol from membranes enter into HDL by an aqueous diffusion, a mechanism by which LCAT generates a concentration gradient favorable to cholesterol efflux. Second, if there are apo A-I particles poor in lipids (pre-β1-HDL), there could be a simultaneous microsolubilization of free cholesterol and phospholipids from plasma membranes with both lipids being directly incorporated into the particle rich in apo A-I and poor in lipid.[44] This theory, together with our findings that HRT increases apo A-I, LpA-I, HDL-phospholipids, and HDL–free-cholesterol, and that all of these parameters are positively correlated between themselves, suggests that HRT may generate a high concentration of lipid-poor apo A-I, which could increase the phospholipid efflux driving cholesterol efflux from plasma membranes to HDL.

The other HDL lipids, cholesterol ester and triglycerides, did not show a significant correlation with the cholesterol efflux. This was not a surprise since HDL–cholesterol ester levels are dependent on other metabolic events like LCAT esterification,[15] transference of neutral lipids between HDL and triglyceride-rich lipoproteins (VLDL, LDL) by CETP,[16] and uptake by the SRBI receptor from hepatocytes.[18] On the other hand, the increase in triglycerides is explained by the known hepatic lipase inhibition produced by estrogens.[45]

PLTP activity showed a behavior different from all others parameters. A late significant decrease of 21% was evident only after 71 days of HRT. Since it has been demonstrated that PLTP shows a positive relationship with blood glucose levels,[46] and that the promoter of the human PLTP gene possesses 5 consensus sequences for the transcription factor Sp1 which is considered a glucose-responsive element, it has been suggested that PLTP is related to glucose metabolism.[20] Taking this information into consideration, the late PLTP decrease could be explained as a secondary effect to the improvement in glucose tolerance that appears to be produced by HRT.[47,48] On the other hand, the possibility that the hormonal effect launched by estrogen-progestin treatment itself might affect and reduce

PLTP activity is not excluded. Interpretation of these results should be made with caution due to the fact that the PLTP decrease was seen only after 71 days (ie, at one time point); however, this finding is in agreement with our recent study done in Finnish women. The study was done in normolipidemic women aged 25 to 34 and 35 to 44 years with normal female hormone status, and the results showed that PLTP activity was significantly lower as compared to that seen among postmenopausal women aged 55 to 64 years.[49] Since PLTP has been implicated as one of the key factors that mediates pre-β-HDL formation, the present decline in PLTP activity suggests that other mechanisms to generate pre-β-HDL along with PLTP might be involved here. Furthermore, estrogen-progestin treatment may generate HDL subpopulations with increased substrate properties for PLTP function, like the present increment of HDL particle triglyceride content. We have demonstrated that when HDL is enriched with triglycerides, the PLTP-mediated HDL conversion and thus pre-β-HDL generation is enhanced.[50] However, further studies are needed to elucidate the mechanism by which estrogens appear to diminish the PLTP activity and its consequences on lipid metabolism. The negative correlations between apo A-I, LpA-I, LpA-I:A-II concentrations, and PLTP activity are very similar to those observed recently in a Finnish population comprising both sexes,[49,51] which suggests that this behavior is not restricted to the population included in the present study. Factors affecting these correlations are rather difficult to interpret. It is known that PLTP remodulates α-HDL, releasing lipid poor apo A-I from the HDL surface. On the other hand, a proteolytic activity associated to PLTP that cleaves lipid-poor apo A-I, transforming them into truncated proteins (amino acids 1 to 196)[52] that appear to have a higher clearance than native apo A-I, has been described.[53] Therefore, we believe that proteolytic activity associated with PLTP might be an important regulator of apoA-I and apo A-I–containing particle levels.

LCAT activity evaluated with exogenous substrates (liposomes) was unchanged by hormonal substitution. Although our findings suggest that this enzyme was not increased, the possibility that LCAT could be more active with its natural substrate, endogenous HDL, cannot be excluded. These findings appear to be contradictory to 2 previous reports that demonstrated an increase in LCAT activity with HRT.[30,54] However, in those studies, the protocol was different since the blood was collected in the combined estrogen-progestin period, whereas in our study it was done at the end of unopposed estrogenic period.

In summary, our study demonstrates that HRT in healthy postmenopausal women modifies the HDL lipid composition, selectively increases the HDL subpopulation that only contains LpA-I, and decreases the activity of PLTP, a key protein regulating HDL levels. The most relevant finding was that HRT increases the plasma capacity to promote cholesterol efflux in vitro. These findings demonstrate that HRT not only increases HDL-cholesterol levels, but also modifies HDL physiochemical properties, possibly optimizing its function in the cholesterol efflux. However, the present study is limited to women from the Chilean population. On the other hand, it is important to point out again that our protocol considered the venous blood sampling at the end of the estrogenic phase, prior to the addition of progestin. For that reason, these results represent mainly an estrogenic effect. Finally, the molecular mechanism by which HRT exerts compositional changes in HDL, on the plasma capacity to carry out cholesterol efflux, and on PLTP activity requires further research.

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