Lipoprotein(a) Levels and Isoforms and Fibrinolytic Activity in Postmenopause - Influence of Hormone Replacement Therapy

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Summary

Epidemiological studies suggest that hormone replacement therapy (HRT) decreases the risk of cardiovascular disease in postmenopausal women via several mechanisms, including modifications in the fibrinolytic system and lipoprotein(a) [Lp(a)] levels. The aim of this study was to examine the influence of the levels and isoforms of Lp(a) on fibrinolytic activity in 91 postmenopausal women in comparison with premenopause and analyze the effect of HRT on those parameters. In postmenopause, an increase in plasma Lp(a) and plasminogen activator inhibitor-1 (PAI-1) levels was found. A significant inverse correlation was observed between Lp(a) or PAI-1 levels and plasmin generation. Plasma samples with low molecular weight (MW) apo(a) isoforms showed higher plasmin inhibition than plasmas with high MW apo(a) isoforms and similar levels of total Lp(a) and PAI-1. HRT induced a significant decrease in Lp(a) and PAI-1 levels and an increase in estradiol levels, as well as an increase in fibrinolytic activity. A significant correlation was found between the percentages of variation in Lp(a) levels and in plasmin generation and between the percentages of variation in PAI-1 levels and in the euglobulin lysis time under HRT. In conclusion, the increase in fibrinolytic activity observed in women under HRT could be explained by two independent mechanisms: (a) the decrease in PAI-1 and (b) the decrease in the inhibition of plasmin generation due to the decrease in Lp(a) levels.

Introduction

An increase in the incidence of coronary heart disease has been found in postmenopausal women (1-4), and epidemiological data suggest that hormone-replacement therapy (HRT) can reduce the risk of cardiovascular disease and mortality in postmenopausal women (3-10). The beneficial effects of HRT on cardiovascular risk could be due to several mechanisms, including modifications in the plasma concentrations of lipoproteins, glucose and insulin, and in blood pressure and the hemostatic system (2, 3, 5, 11, 12).

Modifications of the hemostatic system may play a role in the pathogenesis of coronary heart disease (13, 14). A fibrinolytic hypofunction due to an increase in plasminogen activator inhibitor-1 (PAI-1) has been detected in coronary ischemic disease (13-17), and this increase in PAI-1 has been found to constitute a risk factor for recurrent myocardial infarction (13, 15). An increase in factor VII, fibrinogen and PAI-1 has been reported in postmenopausal women (18-20). Moreover, lipoprotein (a) [Lp(a)] levels have also been found to rise in postmenopausal women (20, 21). These alterations could contribute to the increased risk of cardiovascular disease seen in postmenopausal women.

Lp(a) is a variant of the low-density lipoprotein (LDL) in which apolipoprotein B-100 is covalently linked to a single apolipoprotein (a) [apo(a)] (22, 23). Increased levels of Lp(a) have been considered an independent risk factor linking the pathophysiological processes of atherosclerosis and thrombosis in coronary artery disease (24, 25). However, the mechanism behind these processes is not completely understood. A similarity between the cDNAs of apo(a) and plasminogen (26) and therefore between their protein structures, has been demonstrated. As a consequence of this similar protein structure, Lp(a) could compete “in vitro” with plasminogen for its binding to fibrin and, therefore, Lp(a) might significantly impair physiological fibrinolysis and promote thrombosis (27-33).

Apo(a) shows a size polymorphism, with individual isoforms ranging in apparent molecular weights from about 250 to 800 kD (34, 35), and at least 34 different isoforms of apo(a) have been reported (36). The variation in the size of Lp(a) could result from the number of kringle 4 copies (37). It has also been reported that Lp(a) size heterogeneity is genetically controlled, and an inverse relationship has been observed between the apo(a) isoform size and Lp(a) concentration (34) and between the number of kringle 4 copies and Lp(a) levels (38).

The antifibrinolytic potential of Lp(a) has been studied by several groups, but contradictory results have been reported (39-43). In previous studies (39, 40) we found that the euglobulin lysis time induced by tissue-type plasminogen activator (t-PA) was impaired in subjects with high Lp(a) levels. However, a variable inhibition of fibrinolytic activity was found in plasmas with high Lp(a) levels and it could be due, at least in part, to the presence “in vivo” of different apo(a) isoforms. Hervio et al (44, 45), for example, have shown that inhibition of plasminogen binding to fibrin by apo(a) purified from homozygous or heterozygous subjects was inversely associated with isoform size. In addition, the “in vivo” effect of apo(a) on fibrinolysis has recently been demonstrated in human apo(a) transgenic mice that were resistant to t-PA-mediated thrombolysis (46).

In postmenopausal status, an increase in Lp(a) levels has been detected (20, 21), but no studies on Lp(a) levels and apo(a) isoforms in relation to fibrinolytic activity have been reported. HRT decreases Lp(a) levels (20, 47-52), especially in women with previously increased Lp(a) levels, and increases fibrinolytic activity (20, 53-56). However,
The HRT was applied continuously in the postmenopausal women, and the different parameters were evaluated before, and 3–4 months and 12 months after HRT (Table 2). Of the women receiving HRT one group (Group A) received oral estrogens (estradiol valerate 2 mg/day) plus medroxyprogesterone acetate (Progevera, Upjohn, Kalanamooz, MI, 2.5 mg/day), another group (Group B) received transdermal estradiol (Estraderm, Ciba, Basel, Switzerland, 0.05 mg/day) plus medroxyprogesterone acetate (2.5 mg/day), and the third group (Group C) was given transdermal estradiol, at the same dose as group B.

The premenopausal group comprised 30 healthy women aged from 23 to 50 years (37 ± 9 years) with regular menstruation and without hormonal treatment. In Table 1 the control group comprised 27 premenopausal women with Lp(a) levels <30 mg/dl (Group 3).

All the women were non-smokers. Informed consent was obtained before sample extraction from all the women.

### Materials and Methods

#### Clinical Groups

The postmenopausal group consisted of 91 postmenopausal women (women had not menstruated for at least 1 year before the examination), aged 36 to 65 years, with a mean age of 51.7 ± 6.2 (mean ± SD) years. None of the women had had any hormonal preparation during the 8 weeks prior to the study and none had a history of thromboembolic, severe metabolic, endocrinological or gastrointestinal disease, neoplasm or uncontrolled hypertension.

The 91 postmenopausal women studied were classified according to their plasma Lp(a) levels into two groups. Group 1 comprised 37 women with plasma Lp(a) levels \( \geq 30 \) mg/dl, and Group 2 included 54 women with plasma Lp(a) levels <30 mg/dl (Table 1).

#### Blood Sampling

Venous blood samples were obtained between 8 and 10 am, after overnight fasting. Subjects remained in a sitting position for 20 min before venipuncture. Blood samples were anticoagulated with 0.13 mmol/l trisodium citrate (9:1, vol:vol, blood:anticoagulant), and were immediately centrifuged at 1,500 \( \times g \) for 30 min at 4 °C. Plasma was snap frozen in small portions and stored at –80 °C until the assays were performed in series (within 6 months). Blood serum was used to determine the lipid profile and estradiol levels.

#### Methods

Study of plasma Lp(a) levels and isoforms

Lp(a) levels were determined with an ELISA kit [Macra Lp(a), Terumo] as previously described (39). The assay uses a monoclonal antibody against apo(a) that does not cross-react with plasminogen, and a second polyclonal antibody directed against the apo(a) portion of Lp(a). The assay recognizes all apo(a) isoforms with the same efficiency and does not cross-react with HDL-cholesterol, LDL-cholesterol or VLDL-cholesterol. High total cholesterol or LDL-cholesterol levels do not interfere with the Lp(a) determination (57). The intraassay and interassay variability was 3% and 8%, respectively.

The apo(a) phenotype was determined by agarose gel electrophoresis using the method described by Kamboh et al. (35) with slight modifications, followed by immunoblotting as previously described (58). Apo(a) bands were detected by a double-antibody procedure (Immuno AG) and visualized by histochemical staining for alkaline phosphatase. The sheep antibody to human apo(a) was known to react equivalently with the different isoforms. The isoforms were classified into two groups according to whether they had a molecular weight lower or higher than 625 kD. For correlations the MW of the apo(a) isoform was used.
In the apo(a) heterozygotes, the relative concentration of the two apo(a) isoforms was determined using a scanning densitometer (Shimadzu CS-9000). To assess whether the intensity of the bands was proportional to the amount of Lp(a), increasing volumes of plasma (0.3 to 5 μl) from an individual with a single apo(a) isoform and an Lp(a) level of 77 mg/dl were subjected to electrophoresis and blotted. The area of the band was proportional to the amount of Lp(a) added along all the range studied. To determine whether the relative concentration of the two isoforms remained constant regardless of the total amount of Lp(a) added to the gel, several volumes of plasma (0.6 to 10 μl) from an apo(a) heterozygous individual with a total Lp(a) level of 79 mg/dl were studied. In all cases, the proportionality of each isoform remained constant (data not shown).

Fibrinolytic studies

Euglobulin lysis time was assayed as previously described (39), using fresh plasma. The intraassay variability was 3%.

Determination of t-PA antigen was performed with a commercially available enzyme-linked immunosorbent assay (Imulys t-PA, Biopool). The assay detects free and complexed t-PA with similar efficiency. The intraassay and interassay variability was 4% and 6%, respectively.

PAI-1 antigen was quantified by a commercially available ELISA assay (TintEliza PAI-1, Biopool). The assay detects active and latent (inactive) forms of PAI-1 and complexed PAI-1 with the same efficiency. The intraassay and interassay variability was 3% and 7%, respectively.

The PAI-1 activity assay was performed as previously described (59). One unit of PAI activity is defined as the amount that inhibits 1 IU of single chain t-PA in 15 min at room temperature under the conditions used. The intraassay and interassay variability was 6% and 10%, respectively.

Functional plasminogen activation was measured by a chromogenic substrate method (60). The intra- and interassay coefficients of variation were 5% and 9% respectively.

Generation of plasmin by fibrin-bound t-PA

The effect of Lp(a) on plasminogen activation was determined by measuring the amount of plasmin generated by t-PA in a system with a fibrin surface in the presence of plasminogen, following a modification of the Rouy et al. method (31). Briefly, each well of a solid-phase fibrin 96-well microtiter plate was incubated with single-chain t-PA (sc-t-PA) (20 IU/ml) (Biopool) for 1 h at 37°C. Unbound protein was removed, and 50 μl/well of test plasma diluted 1:3 in 0.05M sodium phosphate, pH 7.4, supplemented with plasminogen (2.4μg) was added in duplicate. After 4 h-incubation at 37°C, the plate was washed, and fibrin-bound plasmin activity was detected by adding 50 μl of 1.5 mM substrate S-2251 (Chromogenix) and measuring the change in the absorbance rate [10⁻⁷ × (ΔA₄₀₅-₄₉₂ /min)]. Plasmin generation is proportional to the amount of fibrin-bound plasminogen, which was added in excess in all the wells, and Lp(a) present in the test plasma can interfere with plasminogen binding, reducing plasmin generation.

Other Laboratory Parameters

Total cholesterol, triglycerides and glucose were determined by an automated method (DAX Technicon).

Estradiol was measured by a commercially available radioimmunoassay (BioMerieux, Marcy l’Etoile), in which the hormone in the sample competed with a fixed amount of 125I-labelled steroid for specific anti-steroid antibodies coated on the inner wall of the tube. The intra- and interassay coefficients of variation for the range of concentrations in our samples were 9.9% and 16.2%, respectively.

Statistical Analysis

Levels of significance between the two groups were determined by the Student t-test, paired t-test and Mann-Whitney non-parametric U test.

Fig. 1 Correlations between total plasma Lp(a) levels and plasmin generation in postmenopausal women prior to hormone-replacement therapy

Comparisons between different groups were performed by ANOVA and multiple comparison tests. The χ² test was used to compare percentages. The correlation coefficient between variables was calculated using the Spearman rank test. A multivariate step-wise regression model was used to validate independent variables that could influence fibrinolytic activity. Values of p <0.05 (two tailed, where applicable) were considered to be significant. All these tests were performed in the statistical package SPSS Release 6.0 for Windows (SPSS Inc., Chicago).

Results

Lp(a) levels, apo(a) Isoforms and Fibrinolytic Parameters in Postmenopause

The plasma Lp(a) levels, apo(a) isoforms and several fibrinolytic parameters, including plasmin generation, were evaluated in the 91 postmenopausal and 30 premenopausal women. Lp(a) levels were significantly higher in postmenopause than in premenopause (26 ± 23 vs 13 ± 9 mg/dl, p <0.0001). The percentage of women with Lp(a) levels ≥30 mg/dl was higher in postmenopausal (41%) than in premenopausal (10%) women. No differences between the post and premenopausal groups with respect to apo(a) isoform size were found (685 ± 70 kD vs 700 ± 68 kD).

In the group of postmenopausal women, a decrease in fibrinolytic activity, evidenced by a prolongation of the euglobulin lysis time and probably due to an increase in PAI-1, was also observed in comparison with the control group (postmenopause vs premenopause: euglobulin lysis time: 170 ± 53 min vs 142 ± 48 min, p <0.05; PAI-1 antigen: 27 ± 17 ng/ml vs 12 ± 6 ng/ml, p <0.01). Moreover a significant correlation between euglobulin lysis time and PAI-1 antigen levels (r = 0.665, p <0.001) was observed.

Postmenopausal Women with High and Normal Lp(a) Levels

The postmenopausal women were grouped according to whether they had plasma Lp(a) levels ≥30 mg/dl (group 1) or <30 mg/dl (group 2) (Table 1). No age differences between the two groups were observed.

In relation to the molecular weight of the major apo(a) isoform, a smaller mean apo(a) size was found in the group of postmenopausal women with high Lp(a) levels than in the group with low Lp(a) levels (Table 1). Moreover, the percentage of women that had low molecular weight (MW) apo(a) isoforms was higher in the group with high levels of Lp(a) (41%) than in the group with normal Lp(a) levels (6%).
In the 91 postmenopausal women, a significant inverse correlation \( (r = -0.385, p < 0.001) \) was observed between Lp(a) levels and plasmin generation (Fig. 1), and between PAI-1 levels and plasmin generation \( (r = -0.487, p < 0.001) \). There was a significant inverse correlation between Lp(a) levels and the size of the major apo(a) isoform \( (r = -0.455, p < 0.001) \). A multivariate analysis revealed that the effect of Lp(a) on fibrinolysis was independent of the PAI-1 levels. On the other hand, to study the influence of the type of apo(a) isoform on the inhibition of plasmin generation, plasmas from postmenopausal women with similar Lp(a) and PAI-1 levels but different apo(a) isoforms were analyzed. Fig. 2 shows that the inhibition of plasmin generation was greater in postmenopausal women with a low MW apo(a) isoform \((n = 12)\) than in those with a high MW apo(a) isoform \((n = 12)\) and similar plasma Lp(a) and PAI-1 levels.

The group of women with a combination of high plasma Lp(a) levels \((230 \text{ mg/ml})\) and high PAI-1 levels \((\geq 20 \text{ ng/ml})\) showed lower plasmin generation \( [6.2 \pm 1.0 \times \Delta A_{405-492/\text{min}}, n = 20] \) than the group with high plasma Lp(a) levels and normal PAI-1 levels \( [7.7 \pm 1.2 \times \Delta A_{405-492/\text{min}}, n = 17] \) and the group with normal Lp(a) levels and high PAI-1 levels \( [7.6 \pm 1.1 \times \Delta A_{405-492/\text{min}}, n = 31] \). The group with normal Lp(a) levels and normal PAI-1 levels \( (n = 23) \) showed higher plasmin generation than the other groups \( [8.6 \pm 2.1 \times \Delta A_{405-492/\text{min}}] \).

**Influence of HRT**

The influence of HRT on the parameters studied is shown in Table 2. HRT induced a significant increase in estradiol levels and a significant decrease in Lp(a) levels. An increase in fibrinolytic activity, due to a significant increase in t-PA levels and a decrease in PAI-1 levels, was observed during HRT (Table 2). No significant modifications in total cholesterol, triglycerides or glucose levels were observed under HRT (Table 2).

There was a significant inverse correlation between the basal (pretreatment) levels of Lp(a) and the variation in Lp(a) after 3–4 months \( (r = -0.725, p < 0.001) \) or 12 months \( (r = -0.980, p < 0.001) \) of HRT. The variation in Lp(a) during HRT was expressed as the difference between the Lp(a) levels after 3–4 or 12 months and the basal levels. The decrease in Lp(a) was higher in the women with basal Lp(a) levels above 30 mg/dl.

The percentage of variation in Lp(a) levels correlated significantly with the percentage of variation in estradiol levels after 3–4 months \( (r = -0.742, p < 0.001) \) or 12 months \( (r = -0.691, p < 0.001) \) of HRT. Furthermore, the percentage of variation in plasmin generation after 3 months of HRT correlated significantly with the percentage of variation in Lp(a) levels \( (r = -0.557, p < 0.01) \) (Fig. 3) but not with the percentage of variation in PAI-1 levels under HRT. However, a significant correlation \( (r = 0.442, p < 0.001) \) was obtained between the percentage of variation of the euglobulin lysis time and the percentage of variation in PAI-1 levels after 5 months of HRT.

In order to compare the results between the different groups of hormone therapies the values were expressed as a percentage, taking the basal value as 100%. The group of oral estrogen + progestogen showed the largest increase in estradiol levels and the largest decrease in Lp(a) levels as well as the highest increase in fibrinolytic activity. A similar increase in t-PA levels during HRT were observed in all the groups studied.
With respect to the influence of progestogen, no significant differences were observed between the group with transdermal estrogen (group C) and the group with transdermal estrogen + progestogen (group B) in any of the parameters studied.

**Discussion**

As in previous studies (20, 21, 47–52), the results of the present one show that Lp(a) levels increase in postmenopause and that HRT reduces this increase. The beneficial effect of HRT on the risk of coronary disease in postmenopause could be due to the decrease in Lp(a) levels.

High Lp(a) levels have been reported to be a major independent risk factor for coronary artery disease (24, 25, 61–63), and the antifibrinolytic potential of Lp(a) in plasma has been studied by several groups, but contradictory results have been obtained (39–43). In the present work, the effect of Lp(a) on plasmin generation was determined by the method of Rouy et al. (31), which was designed to evaluate mainly the effect of Lp(a) on fibrinolysis. We have observed a greater inhibition of plasmin generation in the group of postmenopausal women with high circulating Lp(a) levels than in the group with normal Lp(a) levels. These results regarding the Lp(a) effect on fibrinolysis agree with those previously reported by us and by other authors (39–41). However, in other studies Lp(a) had no effect on fibrinolysis (42, 43). The discrepancy between the results could be explained by the reported occurrence of an apo(a) size polymorphism, because the different apo(a) isoforms could interfere differently with fibrinolysis (42, 43). In fact, it has recently been reported that the influence of Lp(a) on fibrinolysis could depend on the relative concentration of the isoform with higher fibrin affinity (45). In the present study, a significant inverse correlation was observed between Lp(a) levels and plasmin generation and also between Lp(a) levels and the molecular weight of the major apo(a) isoform.

The present study shows, as previously reported by our group and other authors (19, 20), that postmenopause induces an increase in PAI-1 levels and a decrease in fibrinolytic activity. However, our results showed that the effect of Lp(a) on inhibition of plasmin generation was independent of PAI-1 levels. First of all, plasmin generation was significantly lower in postmenopausal women with high circulating Lp(a) levels than in the postmenopausal women with normal Lp(a) levels, even though the two groups had similar PAI-1 levels (Table 1). Moreover, a multivariate analysis revealed that the effect of Lp(a) on fibrinolysis was independent of the PAI-1 levels. On the other hand, in women with similar plasma Lp(a) and PAI-1 levels the inhibition of plasmin generation was greater in those with low MW apo(a) isoform (Fig. 2), which suggests that the inhibition of plasmin generation is influenced by both the total Lp(a) levels and the size of apo(a) isoform. The increase in Lp(a) levels and the decrease in fibrinolytic activity can contribute to the increased risk of coronary disease associated with the postmenopausal state.

As in other recent studies (20, 47–52), our findings show a decrease in Lp(a) levels under HRT. In our study, the decrease in Lp(a) was larger in the women with previous Lp(a) levels above 30 mg/dl, and the percentage of variation in Lp(a) levels correlated significantly with the percentage of variation in plasmin generation after 3 months of HRT (r = –0.557, p <0.01). Moreover, a significant correlation was observed between the percentage of variation in PAI-1 levels and the percentage of variation in the euglobulin lysis time. These results indicate that under HRT the decrease in Lp(a) and PAI-1 levels were associated with increased fibrinolytic activity, and that the plasmin generation technique is more sensitive to evaluate the influence of Lp(a) on fibrinolysis whereas the euglobulin lysis time is more sensitive to changes in PAI-1 levels.

The largest increase in estradiol levels and the largest decrease in Lp(a) levels were observed in the oral estrogen group. The mechanism(s) whereby estrogens might reduce Lp(a) levels is not clear. It is highly likely that the decrease in Lp(a) levels under HRT is due to a reduction in Lp(a) production induced by estrogen in the liver (48), but the regulation of the synthesis, processing, and secretion of Lp(a) is poorly understood.

The influence of the addition of progestogen on cardiovascular disease and the lipid profile has also been studied. It has been concluded (64) that the impact of gestational agents on cardiovascular disease is very much influenced by the dose and duration of therapy with the gestational agent. In the present study, no significant differences in the parameters studied were observed between the group with transdermal estrogen and the group with transdermal estrogen + progestogen.

In conclusion, the data reported here demonstrate that in the postmenopausal state the decrease in plasmin generation observed is influenced by total Lp(a) levels and apo(a) isoform size. Data from this study demonstrate that the use of HRT increases fibrinolytic activity and lowers Lp(a) levels and that these modifications could contribute to the decrease in the risk of coronary ischemic disease in postmenopause.

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