Clinica Chimica Acta 414 (2012) 142–145

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

High density lipoprotein is an inappropriate substrate for hepatic lipase in postmenopausal women

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ARTICLE INFO

Article history:
Received 10 July 2012
Received in revised form 28 August 2012
Accepted 28 August 2012
Available online 4 September 2012

Keywords:
High density lipoprotein
Postmenopause
HDL functionality
Hepatic lipase
Kinetics

ABSTRACT

Background: HDL antiatherogenic effects would not only depend on its concentration but also on its biological quality. Hepatic lipase (HL) action on HDL acts in one of the last steps of reverse cholesterol transport. Cardiovascular risk increases after menopause, however HDL does not decrease even when HL is increased. We evaluated HDL capacity as a substrate of HL in healthy postmenopausal women (PMW).
Methods: We studied 20 PMW (51–60 y) and 20 premenopausal (PreMW) (26–40 y). In fasting serum, lipid–lipoprotein profile and HDL composition were assessed. Optimal assay conditions for HDL/HL *ex vivo* incubation were established. Increasing HDL–triglyceride concentrations (0.015 to 0.20 mmol/l) were incubated with post-heparin plasma obtained from a single healthy donor as a source of HL. Free fatty acids were measured and kinetic parameters calculated: K_m(app), inverse to enzyme affinity, and V_{max}.
Results: HDL composition in PMW exhibits triglyceride enrichment ($p<0.001$). Kinetic analysis revealed higher K_m(app) in PMW [130 (40–380) *vs* 45 (20–91) mmol/l, $p<0.0001$)] correlating directly with HDL–triglycerides ($r=0.7$, $p=0.0001$). Catalytic efficiency, V_{max}/K_m(app) was reduced when compared to controls ($p=0.0001$).
Conclusion: Triglyceride-enriched HDL from PMW constitutes a poor substrate for HL suggesting that this particle may not exert efficiently its antiatherogenic function, regardless of plasma concentration.

1. Introduction

It is well-known that plasma high density lipoprotein cholesterol (HDL-c) levels are inversely associated with the risk of cardiovascular disease, as was clearly demonstrated by the Framingham study [1]. This consistent inverse association suggests that the antiatherogenic function of this lipoprotein would be strongly linked to its concentration. However, it is becoming increasingly clear that the antiatherogenic effects of HDL are not only dependent on its concentration in circulation but also on its biological quality [2]. In fact, it has been suggested that there are circumstances in which an increase in HDL cholesterol is not associated with protection, and conversely, an increase in its protective capacity can be achieved even without a corresponding increase in plasma levels [3]. The reverse cholesterol transport, carried out by HDL, is one of its most studied antiatherogenic functions. By means of this task, HDL is in charge of the removal of the cholesterol excess from peripheral cells, delivering it to the liver for its catabolism. In the last step of the reverse cholesterol transport, hepatic lipase converts triglyceride-rich HDL particles into smaller ones which are recaptured by the liver [4,5]. There is an inverse relationship between HDL levels, especially HDL_2 subfraction, and hepatic lipase activity; this confirms the involvement of the enzyme as phospholipase and triglyceride hydrolase activities on HDL_2 hepatic catabolism. However, alterations in lipoprotein composition and structure could determine a lower HDL catabolism by hepatic lipase.

On the other hand, postmenopausal women present higher cardiovascular risk with an adverse lipoprotein pattern, however HDL-c levels tend to be maintained or slightly decreased, despite having increased hepatic lipase activity mainly due to the estrogen reduction [6,7]. Compositional and structural alterations in HDL could be present in this stage of women life, and this may impact on HDL antiatherogenic properties. In a previous work, we observed an impaired antioxidant action of HDL during LDL oxidation in postmenopausal women, even when HDL did not decrease [8]. In postmenopause, there are no reports concerning the behavior of HDL and its interaction with hepatic lipase, constituting one of the last steps of reverse cholesterol transport. Our aim was to evaluate the capacity of HDL as a substrate of hepatic lipase in postmenopausal healthy women.

Abbreviations: PMW, Postmenopausal women; PreMW, Premenopausal women; PHP, Post-heparin plasma; FFA, Free fatty acids; CETP, Cholesteryl ester transfer protein; K_m(app), Michaelis–Menten constant; V_{max}, Maximum reaction velocity.

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http://dx.doi.org/10.1016/j.cca.2012.08.026

2. Methods

2.1. Subjects

Forty healthy women were studied, 20 were postmenopausal, and who were clinically evaluated and consecutively recruited at the Climacteric Section of a Private Center for Studies in Gynecology in Buenos Aires, with at least 1 y of natural menopause and <10 y of amenorrhea. In all the cases serum levels of FSH > 40 IU/l confirmed the menopausal status. The control group comprised 20 women in reproductive age, with normal physical examination and laboratory tests, recruited consecutively from patients that were attended at the same Center for their routine health check.

None of the women was included when receiving hormonal, hypolipidemic or any other drug known to modify lipid metabolism. Also women with a history of hypothyroidism, diabetes and hepatic or renal disorder were excluded. In no case did alcohol consumption exceed 10 g per day. Post- and premenopausal women were not under regular training exercise. Written informed consent was obtained from each subject before admission to the study, which was approved by the Ethics Committee at the School of Pharmacy and Biochemistry, University of Buenos Aires.

2.2. Samples and analytical procedures

After a 12-h overnight fast, blood samples were collected into dry tubes and serum was separated, kept at 4 °C and used within 24 h for analytical procedures. Cholesterol and triglycerides were measured in serum using commercial enzymatic methods (Roche Diagnostics, Mannheim, Germany) in a Hitachi 917 autoanalyzer. HDL and LDL cholesterol were determined by standardized selective precipitation methods [9,10]. Serum lipid measurements were under good quality control [CV routinely <3%]. Serum apo A-I and apo B were determined by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Within-run and between-day CVs were 1.9% and 2.4% for apo A-I and 1.2% and 2.1% for apo B, respectively.

2.3. Lipoprotein isolation

In other serum aliquot HDL (δ: 1.063–1.210 g/ml) was isolated by sequential preparative ultracentrifugation method at 105,000 ×*g* for 18 h at 10 °C, in a XL-90 Beckman ultracentrifuge, with a type 90 Ti rotor [11]. Addition of EDTA (100 mg/l) in the salt solution used for the adjustment of density was required to minimize lipid peroxidation that may occur during the long centrifugation time. The supernatant was separated and washed once at the same density in order to minimize albumin contamination. The HDL fraction was isolated and filtered through Sephadex G-25 columns (PD-10 columns, Amersham Pharmacia Biotech AB, Sweden), previously equilibrated with Tris HCl 0.01 mol/l, 0.15 mol/l NaCl, pH = 7. 4. This step allows the removal of salts or any other substance that may interfere with the *ex vivo* test incubation. The HDL was layered with nitrogen, stored in the dark at 4 °C and processed within 24 h. Purity and integrity of lipoprotein fraction was tested by agarose gel electrophoresis [12] and, in all cases, a unique band was observed which migrated in electrophoretic mobility corresponding to HDL. Albumin content was quantified using Albumin Tina-Quant (Roche Diagnostics, Mannheim, Germany) in a Hitachi 917 yielded only traces of albumin (<1 mg/dl).

In order to assess HDL composition, cholesterol and triglycerides were measured using the methods previously mentioned, phospholipids were determined following Bartlett [13] and the total protein were quantified using the Lowry method [14]. Total CV for phospholipid determination was 3.1%. Within-run and between-day precision for protein measurement were 3.0% and 4.2%, respectively.

Recovery of the HDL separation method was performed. Isolation of HDL by ultracentrifugation was compared with the isolation by selective precipitation with 40 g/l phosphotungstic acid in the presence of magnesium ions [9]. Assays were carried out in 5 serum samples. Apo A-I, as an indicator of lipoprotein integrity, was measured in total serum and in each corresponding isolated HDL fractions. Percentage of recovery (mean ± SD) after ultracentrifugation was 84 ± 9% and after selective precipitation was 75 ± 8%, p = 0.255. The ultracentrifugation preparative method showed an acceptable HDL–apo A-I recovery.

Table 1
Physical and biochemical features of post- and premenopausal women.

	Postmenopausal women n = 20	Premenopausal women n = 20
Age (years)	52.9 ± 7	33.0 ± 6
Waist (cm)	86.6 ± 10.1[a]	73.9 ± 5.8
Body mass index (kg/m^2)	26.0 ± 3.5[a]	21.4 ± 2.1
Glucose (mg/dl)	97 ± 13	91 ± 13
Total cholesterol (mg/dl)	238 ± 37[a]	192 ± 31
Triglycerides (mg/dl)	155 ± 64[a]	80 ± 36
HDL cholesterol (mg/dl)	59 ± 13	66 ± 14
LDL cholesterol (mg/dl)	152 ± 39[a]	109 ± 31
Apo A-I (mg/dl)	126 ± 15	138 ± 24
Apo B (mg/dl)	120 ± 35[a]	79 ± 19
Apo B/Apo A-I	1.0 ± 0.3[a]	0.6 ± 0.1

Data are means ± SD.
[a] *Vs* premenopausal women, p<0.0001.

2.4. Hepatic lipase source

Post-heparin plasma (PHP) from one healthy donor with normal hepatic lipase activity was used as a source of the enzyme to be used in all the kinetic studies (average activity: 24 ± 2 μmol FFA/ml PHP). Heparin (60 UI/kg body weight) was administered intravenously for the endothelial enzyme release. Ten minutes later, blood obtained by venipuncture of the contra lateral arm was collected in tubes placed in ice and centrifuged at 3500 rpm, 4 °C for 15 min to obtain PHP. The plasma was fractionated into many aliquots to last throughout the study and stored at −70 °C until their use. Activities of other endothelial lipolytic enzymes (lipoprotein lipase and endothelial lipase), present in plasma, were inhibited by the addition of NaCl 1 M into the incubation assay, so lipolytic action is only due to hepatic lipase.

2.5. Lipolysis assay

The isolated and characterized HDL was incubated with the PHP, which provides the hepatic lipase. Increasing HDL–triglyceride concentrations, ranging empirically from 0.01 to 0.30 mmol/l, were applied into the incubation assay with PHP. Bovine serum albumin free of fatty acids (Sigma A-6003) was added in order to capture fatty acids released into the medium, from the triglycerides and phospholipids contained in HDL, and in this way to ensure the continuity of enzyme activity. Heparin (10 mU/ml, final concentration) was also added to promote the enzyme action, and 1 M NaCl to inhibit

Table 2
HDL chemical composition in post- and premenopausal women.

HDL/groups	n	Cholesterol (%)	Triglycerides (%)	Phospholipids (%)	Total protein (%)
Postmenopausal women	20	16.4 ± 2.4[a]	4.8 ± 1.2[b]	21.8 ± 4.7	57.0 ± 5.1
Premenopausal women	20	18.1 ± 3.4	3.5 ± 1.1	22.6 ± 4.5	55.8 ± 5.3

Data are means ± SD.
[a] p<0.05.
[b] p<0.001, *vs* premenopausal women.

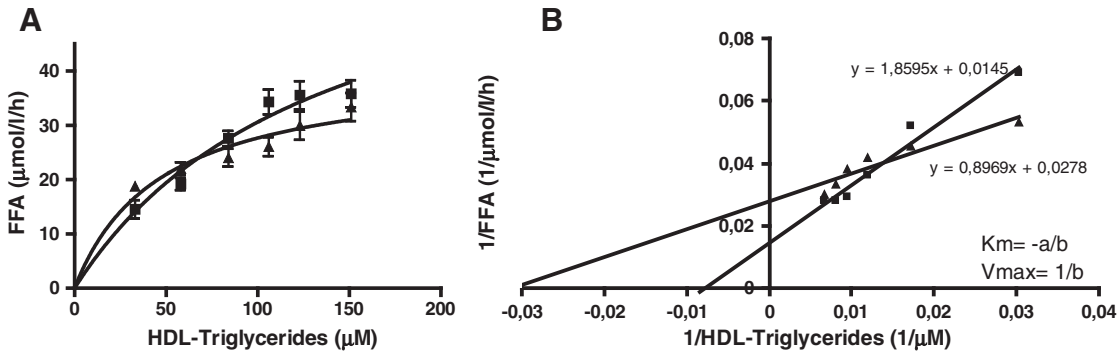

Fig. 1. (A) Michaelis–Menten plot of HDL–triglyceride substrate kinetics for hepatic lipase. (B) Lineweaver–Burk plot of the kinetic data. FFA: free fatty acids; K_m: Michaelis–Menten constant; V_{max}: maximum reaction velocity.

the action of the other endothelial lipolytic enzymes present in the PHP, as discussed above.

Adequate enzyme concentration, incubation time, temperature and type of buffer were previously assayed (data not shown).

Final optimal conditions were, HDL–triglyceride concentration range: 0.015–0.20 mmol/l, PHP aliquot: 50 µl, pH = 7.4, temperature: 37 °C, incubation time: 120 min, albumin concentration: 30 mg/dl, Tris HCl buffer and NaCl concentration 0.01 and 0.15 mol/l respectively and heparin final concentration: 10 mU/ml. After incubation time, the reaction was stopped by placing the tubes in an ice bath and fatty acids (FFA) released, as a product of the hydrolysis, were determined enzymatically by a commercial assay (FA-115, Randox Laboratories, UK). The production of FFA *vs* substrate concentration was plotted and Michaelis–Menten constant (K_m(app), expressed as µmol/l), and maximum reaction velocity (V_{max}, µmol/l/h), were calculated by nonlinear fitting using the SPSS 19.0 software package program (Chicago, IL). Under our experimental conditions the rate of lipolysis was linear for at least 150 min. The amount of substrate was not rate-limiting. Furthermore, the catalytic efficiency was calculated by dividing the V_{max} by the K_m(app) and expressed as h^{-1}.

2.6. Statistical analysis

Results were expressed as mean ± S.D. for normally distributed data and as median (range) for skewed data. Differences between groups were tested using the unpaired Student's *t*-test for normally distributed data and the Mann–Whitney *U*-test for skewed data. Correlations between variables were assessed using the Spearman correlation tests. Differences were considered significant at $p<5\%$.

3. Results

As expected, postmenopausal women (PMW) showed an abnormal lipid–lipoprotein profile as well as a higher obesity degree with abdominal fat distribution in comparison to premenopausal women, $p<0.0001$ (PreMW) (Table 1). There were no differences in HDL cholesterol ($p=0.08$) and apo A-I ($p=0.07$) concentrations between groups. Apo B concentrations and apoB/apoAI ratio were significantly higher in PMW in comparison to PreMW ($p<0.0001$), (Table 1). Table 2 shows the chemical composition of HDL from both groups. HDL in PMW exhibits triglyceride enrichment and cholesterol depletion, when compared to PreMW ($p<0.001$, $p<0.05$, respectively).

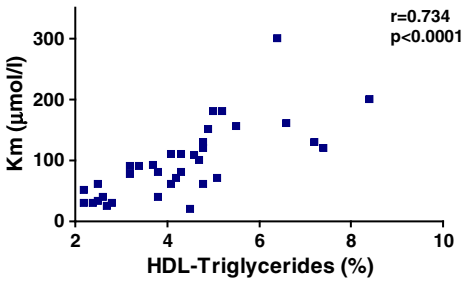

Fig. 2. Correlation between Michaelis–Menten constant [K_m(app)] and HDL–triglyceride content. Linear regression according to Spearman.

Fig. 1 shows the kinetic of HDL incubated with PHP in order to evaluate the HDL capacity as a substrate of hepatic lipase in the *ex vivo* assay. The PMW K_m(app) was increased in comparison to PreMW; regarding V_{max} it was also greater in PMW, however the catalytic efficiency, V_{max}/K_m(app), was reduced when compared to controls (Table 3).

In searching for factors potentially linked to the decreased affinity between the enzyme and HDL, associations with HDL components were estimated. Significant and positive correlations between K_m(app) and HDL–triglyceride content were found, $r=0.734$, $p<0.0001$ (Fig. 2). However, no correlations were observed with the other HDL components.

Table 3
Kinetic parameters from the HDL/hepatic lipase assay.

	Postmenopausal women n = 20	Premenopausal women n = 20
K_m(app) (µmol/l)[a]	130 (40–380)[b]	45 (20–91)
V_{max} (µmol/l/h)[a]	60 (26–96)[b]	37 (17–53)
V_{max}/K_m (h^{-1})	0.47 (0.25–1.13)[b]	0.82 (0.46–1.59)

Data are median (range).
[a] Calculated by nonlinear fitting. K_m(app): Michaelis–Menten constant; V_{max}: maximum reaction velocity. V_{max}/K_m, expresses the catalytic efficiency.
[b] *Vs* premenopausal women, $p<0.0001$.

4. Discussion

Postmenopausal women present an increase risk of cardiovascular disease, though HDL cholesterol does not decrease despite an increase in hepatic lipase activity. In the present study, we investigated the *ex vivo* behavior of HDL particles from postmenopausal healthy women, as a hepatic lipase substrate. It was observed that hepatic lipase showed a lower affinity to HDL from postmenopausal women than to premenopausal HDL, as evidenced by a higher K_m(app), which in turn was associated to an increase in HDL–triglyceride content.

Previous reports studied HDL particles from mice or rabbits [15,16], as well as artificial substrates [17], to assess HDL hydrolysis mediated by purified hepatic lipase. Herein we have assayed human HDL as a hepatic lipase substrate. Post-heparin plasma, employed as

the enzyme source, was obtained from the same healthy donor with a normal hepatic lipase activity. In order to ensure the enzyme specificity, lipoprotein lipase and endothelial lipase were inhibited. Thus, in our assay system the HDL quality as a hepatic lipase substrate was the only variable parameter.

The fact that after menopause HDL level does not decrease despite that hepatic lipase activity increased, has led us to question about the HDL capacity in the enzyme–substrate interaction, and linked to the role of promoting hepatic uptake of lipids [5]. This point has not been studied before.

It must be taken into account the concept that higher HDL due to deficiency of hepatic lipase does not guarantee the efficiency of HDL on its antiatherogenic function [19,20]. This would also support the discordance between the HDL concentration and functionality.

Kinetic assays revealed a higher K_m(app) in postmenopausal women as an indicator of lower enzyme affinity. While V_{max} in this group was also higher, the ratio V_{max}/K_m(app), that reflects the catalytic efficiency of the enzyme–substrate pair, was lower in postmenopausal women in comparison to controls. This result would confirm the poor quality of postmenopausal HDL as a substrate for hepatic lipase, independently of the increased enzyme activity described in postmenopause [18]. As a consequence, HDL would present a reduced catabolism and not entirely complete the influx of cholesterol to the liver.

Evaluation of HDL chemical composition showed an increase in triglyceride content in HDL from postmenopausal women. In addition, this group presented higher plasma triglyceride levels, reflecting VLDL particle accumulation, which promotes lipid exchanges between lipoproteins by means of CETP [21]. As well, CETP activity is also described to be higher in postmenopausal women in comparison to premenopausal women [22], which would contribute to HDL remodeling in circulation. In a previous study carried out in postmenopausal women, we have reported the predominance of HDL rich in triglycerides associated to a lower antioxidant capacity on LDL oxidation [8]. Other authors reported that in the presence of triglyceride enriched HDL particles the reverse cholesterol transport efficiency was decreased [23,24]. Particularly, in the present study, the HDL–triglyceride content correlated positively with K_m(app), suggesting that the HDL–triglyceride enrichment could affect the enzyme affinity towards these particles; perhaps an excess of triglycerides may constitute a steric hindrance. Conversely, according to Rashid et al.'s experiments in rabbits deficient in hepatic lipase, triglyceride enrichment of HDL in the presence of *in vivo* expression of active hepatic lipase resulted in an enhanced HDL clearance [25].

We recognize the need for caution in extrapolation from observations from *ex vivo* assays as that carried out herein. One limitation of this study is that, beyond triglyceride content in HDL, several other factors and/or other HDL components could be involved in the actual HDL–hepatic lipase interaction taking place *in vivo*.

Recent proteomics studies have identified up to 50 less abundant proteins in HDL with many linked to functions [5,26]. Then, HDL apoproteins would also influence the hydrolysis mediated by hepatic lipase. Hime et al., showed that the hepatic lipase had a higher affinity for HDL particles with apo A-II than those containing apo AI [17]. However, Weng et al. demonstrated in transgenic mice overexpressing CETP, that apo A-II inhibits the action of hepatic lipase [27]. Despite the controversy, it must be taken into account that the apoprotein content can also influence the hydrolysis of HDL mediated by the enzyme.

Further studies are necessary to interpret the HDL-hepatic lipase interaction in this group of women, specifically, it remains to analyze HDL behavior against homologous hepatic lipase, the complete HDL protein composition and also to identify different HDL sub-fractions, in order to understand HDL catabolism in menapause. In summary, our results suggest that HDL particles from postmenopausal women are a poor substrate for hepatic lipase, impairing the HDL antiatherogenic function, independently from HDL cholesterol plasma levels.

Acknowledgements

This study was supported by grants of the University of Buenos Aires, B070.

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