Activity of paraoxonase 1 (PON1) and its relationship to markers of lipoprotein oxidation in healthy Slovaks

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Low-density lipoproteins (LDLs), when modified by free radicals derived from artery wall cells, induce atherosclerosis. In contrast to oxidized LDL (ox-LDL), high-density lipoproteins (HDLs) are able to prevent atherosclerosis through a protein with antioxidant properties, paraoxonase 1 (PON1). The purpose of this study was to explore the association between the activity of HDL-associated PON1 and circulating ox-LDL as well as to investigate the relationship between ox-LDL and parameters of lipid profile in thirty Slovaks aged 21–73 years because recent studies have presented controversial results concerning PON1 and its role in LDL oxidation. For determination of circulating ox-LDL sandwich ELISA was used and other lipid parameters were determined by routine laboratory analyses. PON1 activities were assayed by two synthetic substrates — paraoxon and phenyl acetate. Lipid peroxides were determined spectrophotometrically. Of the lipid parameters examined, ox-LDL level correlated positively with total ($P < 0.0001$) and LDL-cholesterol ($P < 0.001$). Triacylglycerols (TAG) ($P < 0.001$), lipid peroxides ($P < 0.01$) and atherogenic index (AI = total cholesterol/HDL) ($P < 0.0001$) were also strongly correlated with ox-LDL. No inverse relationships were observed between ox-LDL and HDL-cholesterol or arylesterase/paraoxonase activities of PON1. Furthermore, it was found that ox-LDL ($P < 0.01$) and lipid peroxides ($P < 0.05$) were significantly higher in men than in women. PON1 arylesterase activity was marginally affected by sex. The results of this study suggest that the anti-atherogenic properties of HDLs are not directly related to their total concentration and that PON1 activity determined towards synthetic compounds (paraoxon and phenyl acetate) reflects no association with markers of oxidative stress. Furthermore, it follows from our results that men are more susceptible to developing atherosclerosis compared to women.

Keywords: atherosclerosis, HDL, ox-LDL, paraoxonase 1

INTRODUCTION

Atherosclerosis is a long-term inflammatory disorder affecting the inner walls of arteries with a great number of predisposing factors such as age, sex, diabetes mellitus, physical inactivity and obesity, cigarette smoking or hypertension. It is characterized by endothelial dysfunction, increased permeability of endothelium to lipoproteins, migration of white blood cells into the walls of arteries, as well as necrosis. LDL particles modified by free radicals are believed to play a critical role in atherosclerosis development. They induce atherosclerosis by stimulating monocyte infiltration and smooth muscle cell migration and proliferation (Mertens & Holvoet, 2001). The presence of oxidatively modified LDL has been evidenced in atherosclerotic lesions of animals such as rabbits (Ylä-Herttuala *et al.*, 1989) as well as in humans. Holvoet *et al.* (1998) found that ox-LDL was related to coronary artery disease. Moreover,

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observations reported by Hulthe and Fagerberg (2002) confirmed an association between subclinical atherosclerosis and ox-LDL by finding that ox-LDL level was related to intima-media thickness.

It is supposed that LDL particles can be protected from free radical-induced oxidation by an HDL linked enzyme, paraoxonase 1 (PON1). PON1 is found in tissues such as liver, kidney, intestine, and also serum (Aviram *et al.*, 1998) and its activity is calcium dependent. It may possess anti-atherogenic and anti-inflammatory properties, resulting from its ability to destroy modified phospholipids and to prevent cumulation of oxidized lipids in lipoproteins, although the precise mechanism of PON1 effect is not yet completely known. Moreover, the reference values of PON1 activities require validation. A variety of external factors, such as antioxidant- or cholesterol-rich diet and lifestyle, can positively as well as negatively influence the activity of human PON1.

Evidence that PON1 is involved in the attenuation of atherosclerosis has mainly been obtained from animal studies. In studies with mice it was shown that PON1-deficient mice were more susceptible to atherosclerosis than wild-type littermates (Gouédard *et al.*, 2003). In human studies, an inverse relationship between PON1 activity and several pathologies associated with atherosclerotic disease such as familial hypercholesterolemia (Mackness *et al.*, 1991; Tomás *et al.*, 2000) or diabetes mellitus type I (Mackness *et al.*, 2002) have been reported.

The aim of this study was to investigate the relationship between the marker of atherosclerosis — ox-LDL — and the activity of the potentially anti-atherogenic enzyme PON1 determined towards paraoxon and phenyl acetate. Thirty Slovak individuals of European lifestyle were investigated.

Additionally, associations between ox-LDL level and parameters of lipid profile were studied in these subjects.

PATIENTS AND METHODS

Patients and sample preparation. Thirty clinically healthy subjects (21 females and 9 males; average age: 41.3 ± 2.9; range: 21–73 years) were studied. All participants gave a written consent to participate in our study. Four individuals were active or occasional smokers.

Blood was taken from vein into commercial tubes (Sarstedt). After centrifugation (10 min at 1200 × g) serum was immediately separated and stored in aliquots at –80°C until use.

Determination of ox-LDL. Circulating ox-LDL in serum was measured by Mercodia Oxidized LDL ELISA kit (Uppsala, Sweden) on a Biorad au-

toanalyser (Roche, Switzerland) according to the producer´s instructions. Results are expressed in arbitrary units as U/L.

Determination of lipid parameters. Total cholesterol, HDL-cholesterol and triacylglycerols were determined by standard biochemical procedures using a Hitachi 911 automatic analyser (Roche, Switzerland). LDL was calculated according to the equation of Friedewald *et al.* (1972).

Determination of PON1 arylesterase activity. For the determination of PON1 arylesterase activity in serum, phenyl acetate was used as a synthetic substrate according to Gan *et al.* (1991) with slight modification. The assay mixture contained 100 µL of 10 mmol/L substrate solution, 5 µL serum and 1 mmol/L $CaCl_2$ (Sigma, USA) in 50 mmol/L Tris buffer (Fluka Chemie, Switzerland), pH 8. Production of phenol was determined spectrophotometrically after 2 min at 270 nm (Biochrom 4060 spectrophotometer, Cambridge, UK). The assay mixture was prepared daily before use. PON1 arylesterase activity was monitored in triplicate and the results are presented as µmol/min per mL. Mean intra-assay and inter-assay coefficients of variation were up to 5% and 8%, respectively.

Determination of PON1 paraoxonase activity. For measurement of PON1 paraoxonase activity in serum, paraoxon (Sigma, USA) was used as a synthetic substrate according to Beltowski *et al.* (2002) with some modifications. After addition of 3.3 mmol/L substrate to the assay mixture containing 10 µL of serum and 1 mmol/L $CaCl_2$ (Sigma, USA) in 20 mmol/L Tris/buffer, pH 8, formation of *p*-nitrophenol was monitored spectrophotometrically at 412 nm for 3 min. PON1 paraoxonase activity was determined in triplicate and all results are presented in nmol/min per mL. Mean intra-assay and inter-assay coefficients of variation for this analysis were 13.3% and 10.9%, respectively.

Determination of lipid peroxides. Serum lipid peroxides were assayed spectrophotometrically (Opton PM2 DL) according to El-Saadani *et al.* (1989). The analysis was based on the ability of lipid peroxides to convert iodide to iodine. Briefly, the reaction was initiated by addition of serum (100 µL) to the assay mixture prepared according to the El-Saadani´s protocol. After 30 min total amount of lipid peroxides was monitored at 365 nm. All results are presented in nmol per mL of sample. Mean intra-assay coefficient of variation for this analysis was lower than 5%.

Statistical analysis. Results are presented as mean ± standard error of mean (S.E.M.) or median (interquartile range, I.R.). Student's unpaired *t*-test and Mann-Whitney U-test were used for statistical analysis of data with a gaussian and nongaussian distribution. $P < 0.05$ was considered to be statisti-

cally significant. StatsDirect® 2.3.7 (Stats Direct Sales, Sale, Cheshire M33 3UY, UK) was used for all correlation analyses. The strength of the association between two investigated parameters was expressed by the Pearson's product moment and Spearman's correlation coefficients.

RESULTS

Table 1 summarizes clinical and biochemical parameters of subjects participating in our study.

We found that the subjects, who were randomly selected for this study, had an increased mean level of total cholesterol (from 4.44 to 8.43 mmol/L) and TAG (from 1.04 to 4.34 mmol/L) compared to reference values. Other mean parameter values of lipid metabolism (HDL- and LDL-cholesterol) (Table 1) were in the range of physiological values (from 0.72 to 2.17 mmol/L and from 2 to 6.6 mmol/L, respectively). Serum ox-LDL level in the study subjects ranged from 45.47 to 152.67 U/L with the mean value of 90.08 ± 4.95 U/L. Median (interquartile range) of the total amount of lipid peroxides was 41.85 (36.08, 77.84) nmol/mL. PON1 arylesterase activity in the subjects ranged from 49.79 to 121.03 μmol/min per mL (mean: 86.04 ± 3.26 μmol/min per mL). Median (interquartile range) of PON1 paraoxonase activity was 61.64 (29.61, 146.35) nmol/min per mL. For ox-LDL, lipid peroxides, and PON1 arylesterase/paraoxonase activities the reference range is not known.

Ox-LDL, lipid peroxides and PON1 activity

The associations between ox-LDL or lipid peroxides and both PON1 arylesterase and paraoxonase activities were examined in the study group by Pearson's correlation and Spearman's rank correlation.

Surprisingly, the values for ox-LDL level as well as lipid peroxides were not correlated with PON1 arylesterase activity (r = –0.207, P = 0.272, n = 30; r = 0.015, P = 0.936, n = 30, respectively). A similar result was also obtained for PON1 paraoxonase activity in relation to ox-LDL and lipid peroxides (r = –0.084, P = 0.657, n = 30; r = –0.146, P = 0.438, n = 30, respectively) where the strength of the association between both parameters was even lower.

Ox-LDL and parameters of lipid metabolism

The relationship between ox-LDL level and parameters of lipid metabolism was also studied. As shown in Table 2, a positive correlation between ox-LDL and total cholesterol was observed (r = 0.755, P < 0.0001, n = 30). Similar associations were found for calculated LDL (r = 0.644, P = 0.0002, n = 30) and TAG (r = 0.609, P = 0.0004, n = 30). Additionally, significant relations were found between ox-LDL and atherogenic index AI (AI = total cholesterol/HDL) (r = 0.756, P < 0.0001, n = 30) or lipid peroxides (r = 0.499, P = 0.005, n = 30). On the other hand, only a negative tendency between ox-LDL and HDL was found (r = –0.343, P = 0.064, n = 30).

Sex and markers of atherosclerosis

Ox-LDL was significantly higher (P = 0.002) in men than in women (Table 3). The mean value of ox-LDL in men was about one third higher compared to women. The amount of lipid peroxides was immensely higher in men in comparison to women (P = 0.019).

As expected, PON1 arylesterase activity was lower in men than in women, but this difference was only marginally significant (P = 0.056). PON1 paraoxonase activity was not different between sexes (P = 0.594).

Table 1. General and biochemical characteristics of subjects.

Data are expressed as mean ± S.E.M. except PON1 paraoxonase activity, lipid peroxides and TAG, which are presented as median (interquartile range, I.R.), the number of subjects in our study = 30, R.V. means reference values determined in our laboratory.

Parameters	Mean ± S.E.M.	Median (I.R.)	R.V.
Age	41.33 ± 2.91		
Serum ox-LDL (U/L)	90.08 ± 4.95		No R.V.
Lipid peroxides (nmol/mL)		41.85 (36.08, 77.84)	No R.V.
PON1 arylesterase activity (μmol/min per mL)	86.04 ± 3.26		No R.V.
PON1 paraoxonase activity (nmol/min per mL)		61.64 (29.61, 146.35)	No R.V.
Total cholesterol (mmol/L)	5.58 ± 0.20		< 5.17
HDL-cholesterol (mmol/L)	1.15 ± 0.06		0.9–1.84
LDL-cholesterol (mmol/L)	3.44 ± 0.19		< 3.5
TAG (mmol/L)		2.15 (1.76, 2.69)	< 2.0

Table 2. Correlation coefficients between ox-LDL and parameters of lipid profile in healthy subjects.

AI (atherogenic index) = total cholesterol/HDL. Pearson correlation was used to evaluate the degree of associations between ox-LDL and total cholesterol. Non-parametric Spearman correlation was used to test the associations between ox-LDL and other variables (HDL- and LDL-cholesterol, TAG, AI and lipid peroxides). $**P < 0.01$, $***P < 0.001$.

Parameter	ox-LDL	
	r	Significance level (P)
Cholesterol		
Total	0.755	***
HDL-	−0.343	0.064
LDL-	0.644	***
TAG	0.609	***
AI	0.756	***
Lipid peroxides	0.499	**

DISCUSSION

Only few studies have found a significant association between the level of ox-LDL and parameters of lipid profile. Sigurdardottir and co-workers (2002) found that ox-LDL is associated with factors of metabolic syndrome such as triacylglycerols, BMI or HDL-cholesterol. Similar results were found by other researchers who reported relationships between ox-LDL and hypercholesterolemia, BMI, age, triacylglycerols, HDL and LDL in selected populations (Holvoet et al., 2001; Kondo et al., 2001).

In this study we report similar findings, although no significant correlation between ox-LDL and HDL was observed. No significant correlation between both parameters in healthy subjects was also observed by Toshima et al. (2000), who used a different type of capture antibodies.

The results of many studies performed in vitro and in vivo suggest that the activity or concentration of PON1 is inversely associated with atherosclerotic processes, in which ox-LDL plays a significant role. In this study we observed no associations between circulating ox-LDLs and arylesterase/paraoxonase activities of PON1 in healthy volunteers. Our inability to observe associations between the activity of PON1 and markers of lipid oxidation could be partly explained by the heterogeneity of the study group with respect to sex, age or profile of lipid metabolism, genetic polymorphism or a small number of individuals included in our study as well as by the fact that PON1 activity determined towards synthetic compounds (in our case phenyl acetate and paraoxon) needs not necessarily correlate with its antioxidant ability.

On the other hand, recent studies have demonstrated that PON1 may not be able to protect LDL from oxidation in vitro as well as in vivo. Teiber et al. (2004) found that purified serum PON1 does not protect LDL against oxidation in in vitro assays initiated with copper or a free radical generator, 2,2'-azobis-2-amidinopropane hydrochloride (AAPH). Similarly, Sampson et al. (2005), who studied associations between PON1 activity and ox-LDL concentration in individuals with diabetes type II, found no relation between these study variables except the relationship of ox-LDL levels and PON1 arylesterase activity in diabetic males. Therefore, that study did not confirm an in vivo beneficial PON1 action in LDL oxidation, either. Because of this discrepancy of results it is necessary to perform a study with a higher number of participants.

As expected, we found that ox-LDL was significantly higher in men compared with women. This finding could support the assumption of a lower incidence of atherosclerosis and cardiovascular diseases in women. It is postulated that estrogens may be responsible for this phenomenon. Because estrogen (estradiol) is a phenol (Parthasarathy et al., 2000), it may participate with other antioxidants in the protection against atherosclerosis. Furthermore, estrogen increases vasodilatation and inhibits the response of blood vessels to injury (Topçuoğlu et al., 2005).

In summary, the present study showed associations between ox-LDL and two traditional risk factors of atherosclerosis (LDL and TAG) as well as atherogenic index (total cholesterol/HDL ratio) in

Table 3. Ox-LDL and other parameters associated with atherosclerosis with respect to sex.

All results are expressed as mean ± S.E.M. except PON1 paraoxonase activity and lipid peroxides, which are presented as median (interquartile range)[†]. Differences in ox-LDL and PON1 arylesterase activity between sexes were analysed by Student's unpaired t-test. For comparison of PON1 paraoxonase activity and lipid peroxides between the two groups, non-parametric Mann-Whitney U-test was used. $*P < 0.05$, $**P < 0.01$.

Parameter	Men	Women	Significance level
	(n = 9)	(n = 21)	(P)
Serum ox-LDL (U/L)	112.16 ± 7.29	80.61 ± 5.16	**
PON1 arylesterase activity (μmol/min per mL)	76.59 ± 6.29	90.09 ± 3.54	0.056
PON1 paraoxonase activity (nmol/min per mL)	55.87 (32.80, 181.61)[†]	80.15 (27.33, 118.74)[†]	0.594
Lipid peroxides (nmol/mL)	139.27 (41.30, 157.72)[†]	38.86 (33.74, 50.81)[†]	*

healthy Slovak volunteers. Moreover, it also confirmed the concept that men are more susceptible than women to ox-LDL production as well as lipid peroxides formation and therefore to development of atherosclerosis. Additionally, the results of this study showed that PON1 activity towards synthetic substrates does reflect no relationship to oxidative stress characterised by its markers (ox-LDL and lipid peroxides). However, this finding needs further investigation.

Acknowledgements

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