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PON1 Paraoxonase Activity is Reduced During HDL Oxidation and is an Indicator of HDL Antioxidant Capacity

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The aim of this study was to investigate the effect of HDL oxidation on PON1 paraoxonase activity. Also, we were interested in investigating the mechanism by which PON1 could be inactivated and the correlation between its enzymatic activity and the antioxidant properties of HDL. Three different oxidation systems were used for the HDL oxidation: (1) oxidation induced by THP1 cells, (2) oxidation induced by copper ions at a concentration 10 μM, and (3) oxidation induced by $^{\bullet}OH$ and $O_2^{\bullet -}$ oxygen free radicals produced by γ-radiolysis. HDL oxidation was followed by the measurement of lipid peroxide formation, and PON1 activity was determined by measuring the rate of paraoxon hydrolysis. Our results show that HDL oxidation is accompanied by a reduction in the PON1 paraoxonase activity. The extent of PON1 inactivation depends both on the extent of HDL oxidation and on the oxidation system used. The rates of HDL oxidation and PON1 inactivation were significantly correlated ($r = 0.93$, $p < 0.0054$). Our results show that oxidized HDL loses its protective effect toward LDL oxidation. The antioxidant action of HDL towards LDL oxidation and the degradation of PON1 paraoxonase activity were significantly correlated ($r = 0.95$, $p < 0.04$).

Keywords: HDL; Lipid peroxidation; PON1; Paraoxonase activity; Free radicals

INTRODUCTION

The antioxidant effect of HDL is related, in part, to the PON1 enzyme.[1,2] However, the exact mechanism by which PON1 exerts its protective effect is not well established and it has been proposed that this antioxidant effect could be associated with the peroxidase-like activity of PON1.[2] Thus, by hydrolyzing preformed lipid peroxides, PON1 can delay or inhibit the initiation of oxidation induced by metal ions. It is noteworthy that preformed lipid peroxides are essential for the initiation of LDL or HDL peroxidation induced by copper ions.[3] The PON1 activity has been demonstrated to be reduced in different pathologies associated with atherosclerosis such as diabetes, familial hypercholesterolemia, chronic renal failure, post-myocardial infarction and also with smoking and aging.[4–8] However, the real mechanism for PON1 inactivation remains a subject of debate. Even though PON1 activity was demonstrated to be reduced in these physiopathological conditions, less interest was given to investigating the relationship between its enzymatic and antioxidant activities.

This study is aimed to examine the effect of HDL oxidation on PON1 paraoxonase activity. We were also interested in the effect of the oxidant system on the kinetics of PON1 inactivation and the correlation between its enzymatic activity and antioxidant property of HDL.

METHODS

Subjects

Ten subjects (6 F and 4 M), aged between 25 and 39 years, participated to this study. They were all

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healthy volunteers and normolipidemic. Blood pressure profile, glycemia, fibrinogen level, lipid and coagulation profiles were within normal ranges. The study was approved by the Ethics Committee of the Institut Universitaire de Gériatrie de Sherbrooke, and all subjects gave written informed consent.

LDL Isolation

To avoid PON1 inactivation by EDTA, blood was collected in heparin from each donor. All precautions were taken to prevent lipoprotein modifications during the procedure. LDL ($1.019 < d < 1.063$) and HDL ($1.063 < d < 1.19$) were isolated by 2 h of ultracentrifugation according to the method of Sattler *et al.*[9] Proteins were measured by commercial assay (Pierce method, Rockford, IL, USA).

Cell-induced HDL Oxidation

Monocytic THP1 cells were obtained from American Type Culture Collection (TIB-202) and cultured in RPMI 1640 medium containing 20 μmol/l 2-mercaptoethanol, 10% fetal serum and 40 μg/ml gentamycin. For HDL oxidation, cells were washed three times with Ham's F-10 medium to remove serum and were plated at 3×10^6 cells/35 × 10-mm dishes in 1 ml Ham's F-10 medium supplemented with gentamycin (40 μg/ml). Phorbol 12-Myristate 13-Acetate (PMA) was used at a concentration of 1 μM for THP1 cells stimulation. HDL were added to the culture medium to a final concentration of 0.4 mg protein/ml. Incubation was then carried out for 0–48 h at 37°C in a humidified atmosphere containing 5% CO_2. At the end of each incubation time, samples were centrifuged (at 500*g* for 10 min at 4°C) and the supernatant was analyzed immediately for lipid peroxide formation.

Copper-induced HDL Oxidation

HDL were incubated for the indicated periods at 37°C in 10^{-2} mM in sodium phosphate buffer pH 7, containing 10 μM cupric sulfate. Oxidation reactions were stopped by simply cooling in an ice bath without EDTA addition and the lipid peroxides formed were measured immediately.

γ-Radiolysis-induced HDL and/or LDL Oxidation

γ-Irradiations were carried out in a ^{60}Co Gamma cell 220 (Atomic Energy of Canada Ltd.). Irradiations were performed as previously described.[10] The initial dose-rate was 0.086 Gy/s as determined by Fricke.[11] In brief, HDL and/or LDL (2 ml in each tube) were irradiated in aerated aqueous solutions containing 10^{-2} M sodium phosphate buffer at pH 7.0. Under these conditions, the main radical species formed are hydroxyl and superoxide anion radicals with respective yields of 2.8×10^{-7} mol J^{-1} and 3.4×10^{-7} mol J^{-1}.[12]

Assessment of LDL and HDL Oxidation

HDL and/or LDL peroxidation was followed by measuring lipid peroxide formation according to the method of el-Saadani *et al.*[13] The working reagent was prepared in our laboratory as described.[14] Lipid peroxidation was also measured by monitoring the absorption at 234 nm.[15] Results obtained were in the same range as those obtained for lipid peroxide formation. These data are not shown in order to avoid redundancy.

Evaluation of the Antioxidant Effect of HDL Towards LDL Oxidation

The antioxidant effect of HDL towards LDL oxidation was evaluated by subtracting the amounts of lipid peroxide formed in HDL oxidized alone from those formed in the mixtures (LDL + HDL) as already described.[16] The oxidized form of HDL (oxHDL) was also tested for its antioxidant effect. Native HDL (nHDL) as well as oxHDL were mixed with LDL at a ratio of LDL/HDL 0.1/0.2 mg protein/ml.

Electrophoretic Mobility

The antioxidant effect of HDL towards LDL oxidation was also evaluated by measuring the electrophoretic mobility of LDL submitted to oxidative conditions in the absence or presence of HDL (nHDL or oxHDL). Electrophoretic mobility was measured using 5 μl of each sample as already described.[17]

HDL PON Activity

PON1 paraoxonase (p.ase) activity was measured by the initial velocity of paraoxon hydrolysis, at 25°C, to yield *p*-nitrophenol at 412 nm ($\varepsilon_{412\,nm} = 18290\,M^{-1}\,cm^{-1}$).[17] A PON1 activity of 1 U/l was defined as 1 μM of *p*-nitrophenol formed per min. Results are expressed in U/mg protein. The phenotype of each subject was determined by an enzymatic method.[18,19] Six individuals were of phenotype A, three were AB and one was of phenotype B.

Statistical Analysis

Results are presented as pooled data from 10 independent experiments (means ± SD), performed in duplicate. Mean values were compared using the student's *t*-test, to detect significant differences.

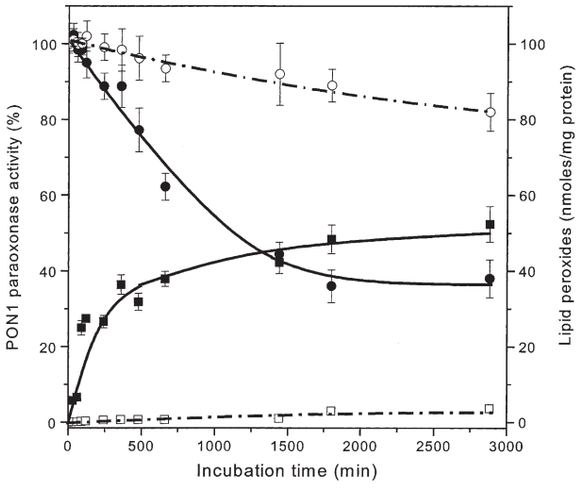

FIGURE 1 Lipid peroxide formation and PON1 p.ase activity during HDL oxidation induced by THP1 monocyte-like cells. See text for details. (■, □) Lipid peroxides formation and (●, ○) PON1 p.ase activity in HDL incubated with THP1 cells or in medium alone, respectively. Each value is the mean ± standard deviation of 10 different experiments.

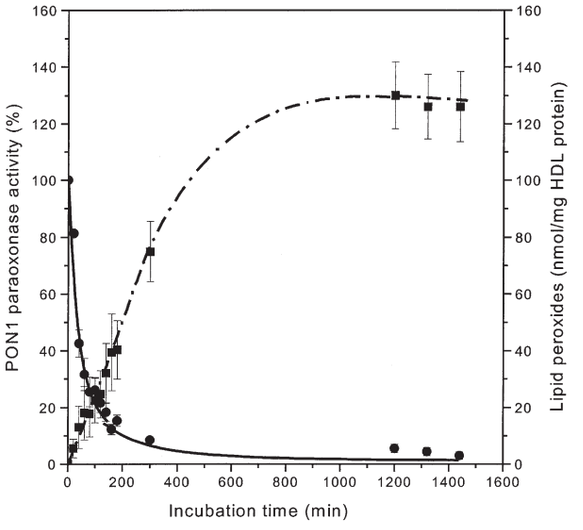

FIGURE 2 Lipid peroxide formation and PON1 p.ase activity during HDL oxidation induced by copper ions. See text for details. Lipid peroxide formation (■), PON1 p.ase activity (●). Each value is the mean ± standard deviation of 10 different experiments.

The relationships between the parameters were determined by regression and correlation analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

THP1 Cell-induced HDL Oxidation

We initially studied oxidation of HDL induced by THP1-cells and evaluated the activity of PON1 to hydrolyse paraoxon (p.ase activity) at different stages of HDL oxidation. LP formation increased with the incubation time ($\nu = 0.19 \pm 0.03$ nmol/mg protein/min) and tapered off between 30 and 48 h (Fig. 1). HDL incubated in medium alone without cells were used as reference and very low amounts of LP were detected in this case (Fig. 1). In parallel, we studied the activity of PON1 towards paraoxon hydrolysis. Incubation of HDL with THP1 cells resulted in a diminution in the PON1 activity (Fig. 1). PON1 alteration was expressed as a percent reduction in its p.ase activity with respect to its initial value. PON1 p.ase activity decreased with a rate of -0.05%/min and lost approximately 65 ± 5% of its total activity after 30–48 h of HDL incubation with cells. This corresponds to the incubation time of maximum lipid peroxide formation. A reduction in PON1 activity was also observed when HDL were incubated with medium Ham's F-10 alone (without cells). Although this was to a lesser extent (approximately a decrease of 18% after 48 h) and with an activity decrease-rate of -0.91×10^{-2}/min. It should be noted that Ham's F-10 medium contains traces of ion metals: 10 nM cupric sulfate and 3 μM ferrous sulfate (according to the product notice).

Copper Induced HDL Oxidation

HDL were incubated with copper ions at a concentration 10 μM. Oxidation was followed as a function of the incubation time (0–24 h). Figure 2 shows LP formation curves obtained during HDL oxidation. Incubation of HDL with Cu^{2+} induces a sharp increase in LP formation for the first minutes of incubation with a rate of -0.25 ± 0.03 nmol/mg protein/min). This value is slightly higher than the value obtained in the presence of THP1 cells (Table I). Also, the maximum LP formed is 2.5 fold higher than that in the presence of cells

TABLE I Kinetic parameters of HDL oxidation and PON1 p.ase activity as a function of oxidant system

	ν_{LP} nmol/mg/min	− % PON (%/min)	$[LP]_{plateau}$ (nmol/mg protein)	%PON remaining
THP1	0.19 ± 0.03	−0.05	51.93 ± 4.72	35.37 ± 5.07%
Medium alone	n.a.	-0.91×10^{-2}		82.12 ± 4.94%
Copper (10 μM)	0.25 ± 0.03	−1.35 ± 0.09	127.12 ± 13.52	3.81 ± 2.12%
γ-(−vit.E)	4.69 ± 0.04	−4.31 ± 0.02	112.56 ± 8.36	51.92 ± 5.84%
γ-(+vit.E)	2.58 ± 0.19	−1.72 ± 0.03	n.a.	65.25 ± 4.97%

(127.12 ± 13.52 nmol/mg protein *vs.* 51.93 ± 4.72 nmol/mg protein). Moreover, the PON1 p.ase activity decreased dramatically upon incubation of HDL with copper ions. The velocity of PON1 activity decrease was about −1.35 ± 0.09%/min, which is 240 times higher than in the presence of THP1. At the longer incubation time, 6–24 h, less than 3.8% of the initial PON1 p.ase activity was still expressed by HDL.

HDL Oxidation Induced by $^{\bullet}OH$ and $O_2^{\bullet -}$ Free Radicals Produced by γ-radiolysis

For better elucidating the mechanism of loss of PON1 p.ase activity during HDL oxidation, we used an oxidation system, which contained neither cells nor metal ions. We thus applied the method of γ-radiolysis of water to produce $^{\bullet}OH$ and $O_2^{\bullet -}$ oxygen free radicals with respective yields equal to 2.8×10^{-7} and 3.4×10^{-7} mol J^{-1}. Phosphate buffer solutions were prepared from tridistilled water and thereafter treated with chelex and the ascorbate test was applied to ensure an absence of metals ions.[20] HDL, at a concentration 0.4 mg/ml in 10^{-2} M phosphate buffer (pH 7.0), were submitted to γ-irradiation at a dose-rate of 0.086 Gy/s with total final doses between 0 and 200 Gy. Results are expressed as lipid peroxide formed and PON1 p.ase activity as a function of irradiation time in order to permit the comparison with results obtained with THP1 cells or copper-induced HDL oxidation. LP formation and PON1 activity degradation increased dramatically with radiation time (radiation dose) corresponding respectively to a rate constant equal to 4.69 ± 0.04 nmol/mg protein/min and −4.31 ± 0.02%/min (Fig. 3). These values are very high when compared to those obtained during chemical oxidation (incubation with copper) or in THP1 cell-induced HDL oxidation.

Even if the rate of HDL oxidation is very high, when compared to oxidation induced by copper, the maximum LP formed at the higher radiation dose is low, 112.56 ± 8.36 vs. 127.12 ± 13.52 nmol/mg protein. Moreover, PON1 p.ase activity decreased by only 48% at the maximum radiation time applied *vs.* about 94% induced by copper oxidation after 24 h of incubation. Enrichment of HDL with α-tocopherol (0.64–1.95 α-tocopherol per HDL particle) reduced lipid peroxide formation rates and PON1 activity degradation (Fig. 3, dot-line). Table I summarizes the principle results obtained for HDL lipid peroxidation and PON1 p.ase activity degradation rates under the different conditions cited above.

We subsequently investigated whether there is a relationship between the kinetics of oxidation products formed within HDL and the PON1 decreasing activity. Our results shown a strong and significant correlation between the rates of LP formation and percent decrease of PON1 p.ase inactivation ($r = 0.93$, $p < 0.0054$).

PON1 (p.ase) Activity Degradation and HDL Antioxidant Properties

In the second part of our study, we were interested in investigating the effect of PON1 p.ase activity degradation on the antioxidant protective effect of HDL on LDL oxidation. Thus, we initially studied the antioxidant effect of HDL under conditions where lipid peroxidation was initiated only by oxygen free radicals produced by γ-radiolysis. Thereafter, we studied the properties of HDL, in their native (nHDL) and oxidized form (oxHDL), towards LDL oxidation induced in the same conditions i.e. γ-radiolysis. HDL oxidized by THP1 cells or by copper ions were dialyzed, before incubation with LDL, to ensure complete elimination of free copper ions contained in the oxidation medium.

Oxidation of HDL reduces their ability to protect LDL (Fig. 4A). This reduction in HDL antioxidant activity was dependent on the extent of their oxidation before incubation with LDL as judged by the value of plateau of the curves (Figs. 1–3). Considering that the maximum of HDL oxidation corresponded, in our case, to the maximum reached for LP formation we can then suggest that the more oxidized the HDL, the weaker the antioxidant effect. Thus, HDL incubated for 20–30 h with copper ions show less antioxidant protection than HDL oxidized in the presence of THP1 cells for the same time or

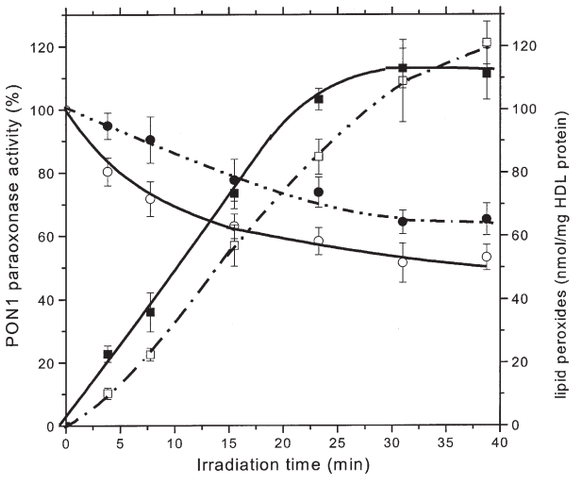

FIGURE 3 Lipid peroxide formation and PON1 p.ase activity during HDL oxidation induced by $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals generated by γ-radiolysis. HDL were used as native or after α-tocopherol enrichment (α-Toc/HDL particle was increased by about 3 fold). (■, □) Lipid peroxide formation and (●, ○) PON1 p.ase activity in native and α-tocopherol-enriched HDL, respectively. Each value is the mean ± standard deviation of 10 different experiments.

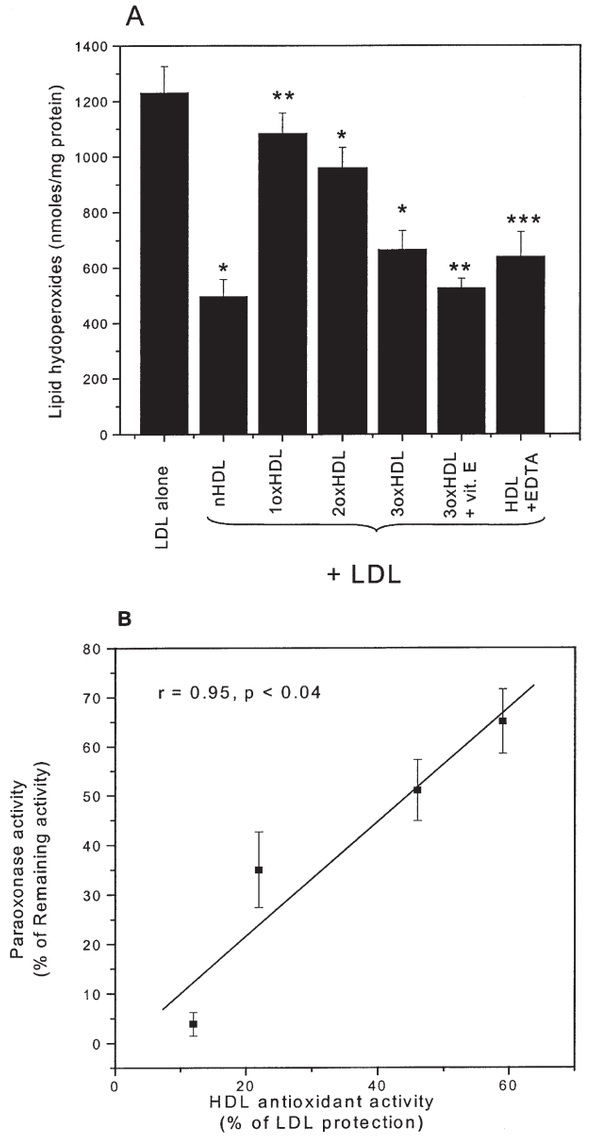

FIGURE 4 (A) Effect of HDL oxidation on its antioxidant activity towards LDL oxidation. LDL were oxidized alone or in presence of native (nHDL) and oxidized HDL (oxHDL). 1oxHDL, 2oxHDL, 3oxHDL corresponded to HDL fully oxidized (at the plateau) by copper ions (10 μM), THP-1 monocyte-like cells or by γ-radiolysis. 3oxHDL + vit.E corresponded to fully oxidized α-tocopherol enriched-HDL (38 min of irradiation). Results are given as mean SD ($n = 5$), *$p < 0.01$, **$p < 0.03$, ***$P < 0.04$. (B) Correlation of PON1 p.ase activity and HDL antioxidant protection towards LDL oxidation. Results are given as mean ± SD ($n = 10$).

than HDL completely oxidized (for 38 min) by γ-radiolysis. The percentage of HDL protection against LDL oxidation were, respectively equal to 12.03 ± 6.10 ($p < 0.03$), 22.03 ± 5.93 ($p < 0.01$) and 46.01 ± 5.61 ($p < 0.01$), (expressed as a percentage by which LDL oxidation was reduced) (Fig. 4A). The antioxidant effect of nHDL and oxHDL was strongly and significantly correlated with the associated PON1 p.ase activity ($r = 0.95$, $p < 0.04$) (Fig. 4B).

TABLE II Oxidative modification of LDL as determined by the electrophoretic mobility

Band	Relative electrophoretic mobility compared to LDL control
Control LDL (0–Gy)	1
LDL alone (200–Gy)	2.5
LDL + nHDL (200–Gy)	1.2
LDL + ox1HDL (200–Gy)	2.3
LDL + ox2 HDL (200–Gy)	1.9
LDL + ox3HDL (200–Gy)	1.6
LDL + ox3HDL + vit.E (200–Gy)	1.3
LDL + EDTA-HDL (200–Gy)	1.8

Data are expressed as the relative electrophoretic mobility of each band compared to that of the control (native) LDL band. LDL were oxidized alone or in the presence of native or modified HDL. Legends are the same as for Fig. 4.

Study of the antioxidant protective effect of HDL in their native and oxidized forms towards Apo B apolipoprotein, forming LDL, was also studied by measuring the electrophoretic mobility of LDL under the different conditions cited above. The results obtained have the same tendency as for lipid peroxide formation (Table II). Apo-B modification increased with the extent of HDL oxidation. oxHDL oxidized by copper ions gave low protection when compared to oxHDL oxidized by cells or by γ-radiolysis even if they were all oxidized to their maximum.

DISCUSSION

Evidence suggests that the antioxidant effect of HDL is principally assumed by one of the HDL-associated enzymes, PON1. PON1 confers its antioxidant effect also towards HDL during their oxidation.[21–24] The question thus arises as to the antioxidative property of PON1 during oxidative stress conditions under which HDL are also oxidized. Moreover, the PON1 antioxidant protective effect tested *in vitro* during HDL or LDL oxidation initiated by copper ions as initiators of oxidation is complicated.[25] Indeed, during these experiments PON1 activity may be affected by Ca^{++} displacement induced by copper ions.[26] Thus, we have attempted to address this question by investigating the effect of HDL oxidation, induced under different conditions, on PON1 enzymatic activity (paraoxonase activity) and its effect on the antioxidant property of HDL.

PON1 activity (arylesterase activity) has already been studied under oxidative conditions and was also shown to be reduced in the presence of oxLDL and/or during oxidation of LDL induced by copper.[27] However, it is more judicious to investigate the activity of PON1 directly in its natural site within HDL. Indeed, PON1 is entirely complexed to

these lipoproteins in serum.[28] Moreover, under oxidative stress conditions, HDL constitute a target for oxidative modifications that may affect their antioxidant properties. It should also be noted that PON1 activity is strongly dependent on its stability, which is enhanced in a phospholipid environment and in association with Apo AI apolipoprotein.[21,29] Thus, due to the difference in lipid/protein composition between LDL and HDL, the kinetics of PON1 inactivation may be different when it is associated with HDL or simply used as a supplement to solutions of LDL.[30]

In our oxidation systems, PON1 p.ase activity degradation, during HDL oxidation, occurs in the presence of copper ions as well as in their absence (by γ-radiolysis). PON1 p.ase activity was completely inhibited in HDL isolated in the presence of EDTA. Although the antioxidative properties of these HDL was only slightly reduced, 59.09 ± 5.12 ($p < 0.01$) vs. 48.04 ± 7.23 ($p < 0.04$), respectively. This result clearly suggests that PON1 may not be the only mechanism by which HDL inhibits LDL oxidation.[31] Our results show a significant and positive correlation between the rate of PON1 p.ase inactivation and lipid peroxidation rates ($r = 0.93$, $p < 0.0054$) which demonstrate that lipid peroxidation products contribute to the PON1 inactivation. However, even for a slight difference in the amount of lipid peroxides formed, at the plateau, during HDL oxidation induced by copper or by γ-radiolysis (127.0 ± 13.52 vs. 112.56 ± 8.36 nmol/mg protein, respectively) loss of PON1 p.ase activity was more dramatic in copper-induced HDL oxidation system. Accordingly, it is possible that PON1 inactivation would also result from a conformational change and/or decreased stability due to Ca^{++} displacement.[32,33] Thus, in copper-induced HDL oxidation systems, PON1 inactivation could be due to both Ca^{++} displacement, induced by copper, and also by lipid peroxides formed during HDL oxidation. The effect of copper ions on PON1 could not be limited to Ca^{++} displacement but may also interact with amino acids in the active site. Besides this, we can not exclude that the inactivation of PON1 towards its antioxidant effect may result from direct free radicals attack on the amino acid groups localized at the active site of the enzyme such as Trp280 as suggested by Aviram *et al.*[26] Oda *et al.* demonstrated recently that an alteration of cysteine amino acid on the N-terminal portion of Apo A1 could induce a reduction in the PON enzymatic activity.[34] On the basis of this, it is probable that the reduction in PON1 p.ase activity during HDL oxidation may be associated with Apo A1 oxidation. Although, the effect of lipid peroxidation on PON1 inactivation should be greater than Ca^{++} displacement or other mechanism affecting PON1 activity and this is supported by the strong and significant correlation between rates of lipid peroxide formation and PON1 inactivation.

α-Tocopherol-enriched oxHDL (3ox-HDL-vit. E) showed an increased antioxidant activity towards LDL oxidation when compared to 3oxHDL (without α-tocopherol enrichment). Analysis of the α-tocopherol contained within 3ox-HDL, the oxidized form of α-tocopherol-enriched HDL, showed that α-tocopherol content was still higher, about 2-fold, in comparison to nHDL (results not shown). Thus, it is possible that α-tocopherol was transferred from HDL to LDL during incubation and thus participates in the protection of LDL against oxidation.

Our results show a significant and positive correlation between PON1 p.ase inactivation and the antioxidant ability of HDL to protect LDL ($r = 0.95$, $p < 0.04$) (Fig. 4B). Although, it is well known that the antioxidant activity of HDL is due only in part to the PON1 and other HDL components participate in this activity among them, platelet activating factor acetylhydrolase (PAF-AH), lecithin-cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP) and Apo A1.[35] Thus, our results suggest an alteration of all HDL components during HDL oxidation and these alterations are with the same extents than those induced to PON1. Thus, the percentage of PON1 inactivation can be used as an indicator of HDL antioxidant capacity.

In summary, HDL oxidation, as evaluated by lipid peroxide formation, is accompanied by an alteration in the PON1 p.ase activity and reduction in the antioxidant properties of HDL towards LDL oxidation. The degradation of PON1 p.ase activity and loss of HDL antioxidant ability was strongly and significantly correlated. The loss of PON1 activity in oxidized HDL may be relevant *in vivo* and could have a dramatic impact in the development and progression of atherosclerosis in patho-physiological situations where plasma paraoxonase activity was shown to decrease.

Acknowledgements

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