HDL Oxidation Compromises its Influence on Paraoxonase-1 Secretion and its Capacity to Modulate Enzyme Activity

Sara Deakin, Xenia Moren, Richard W. James

Objective—The purpose of this study was to analyze the consequences of HDL oxidation for paraoxonase-1 metabolism and function.

Methods and Results—HDL was oxidized with AAPH, copper ions, and hypochlorite. Secretion studies were performed using human paraoxonase-1–transfected cells lines and primary rat hepatocytes. Stability studies were performed with recombinant paraoxonase. Conditioned medium had significantly reduced paraoxonase-1 when Cu or AAPH-oxidized HDL was the acceptor complex (P<0.01); reduction was dose-dependent on the degree of oxidation. Oxidized HDL had a reduced capacity to stabilize/improve activity of secreted paraoxonase-1. Reduced secretion could not be attributed to enzyme inactivation by lipoperoxides, reduced binding affinity of HDL, or oxidation of the lipid component alone. Hypochlorite oxidation of HDL did not modify HDL-mediated paraoxonase-1 release, but activity of HDL-associated paraoxonase-1 was particularly sensitive to such treatment.

Conclusions—AAPH and copper, but not hypochlorite, oxidation of HDL compromises its ability to promote release of paraoxonase-1 and stabilize enzyme activity. HDL-associated paraoxonase-1 is highly sensitive to hypochlorite. Reducing paraoxonase-1 renders HDL susceptible to oxidation, which may compromise HDL function. It provides a novel example at the HDL level of the detrimental effects of oxidative stress, and underlines the need for further evaluation of the consequences of HDL oxidation. (Arterioscler Thromb Vasc Biol. 2007;27:1146-1152.)

Key Words: HDL ■ oxidative stress ■ myeloperoxidase ■ hepatocyte ■ lactonase

High density lipoprotein (HDL) has a well established protective influence against coronary disease. Current interest is focused on the under-exploited therapeutic potential of the lipoprotein. This in turn has drawn attention to the means by which HDL affords cardioprotection, where a number of plausible pathways have been demonstrated.1 A corollary to this line of investigation is to define and understand processes that hinder or compromise HDL function.

Oxidative stress is detrimental to blood lipid metabolism and accorded a major role in the atherosclerotic process.2 The impact is usually considered in terms of oxidative changes to low density lipoproteins (LDL). However, HDL function is also compromised by oxidative stress, as shown over a decade ago for HDL lipid oxidation3 and more recently with myeloperoxidase-mediated oxidation where hypochlorite is implicated.4 Moreover, in comparison to LDL, HDL has a weak complement of antioxidants such as vitamins.5 The lipoprotein can bolster its antioxidant capacity by acquiring peptides capable of such activity. In this context, an important role has been attributed to the enzyme paraoxonase-1 (PON1). Its ability to modulate atherosclerotic lesion development has been demonstrated in animal models and linked to its antioxidant activity.6,7 Conversely, HDL deficient in PON1 is transformed into a prooxidant, proinflammatory complex.8

We recently proposed a mechanism by which HDL can acquire its complement of PON1.9 Association of HDL with the hepatocyte allows transfer of PON1 from a cell membrane location to the lipoprotein. Factors that interfere with this process can influence the PON1 concentration of HDL.9 The present study indicates that the process is sensitive to the oxidative status of HDL. Oxidation of HDL decreases its ability to remove PON1 from cells, modifies its capacity to increase activity of and stabilize desorbed enzyme, and inactivates PON1. It suggests an additional mechanism by which oxidative stress can influence HDL function and impact on the atherosclerotic process.

Methods

Oxidation of HDL

HDL was oxidized under standardized conditions using 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH, 10 mmol/L10) to give oxidized HDL (HDLox) containing 50 to 60 nmoles lipoperoxides/mg HDL protein for the majority of studies reported herein.
unless otherwise indicated. This corresponds to concentrations of HDL-associated LPO that have been reported in high risk populations and studies of cholesterol efflux. Copper sulfate was occasionally used to oxidize HDL. Sodium hypochlorite treatment of HDL was performed as described.

**Release of PON1 From Cells**

Studies of PON1 secretion were based on a Chinese hamster ovary (CHO) model stably transfected with human PON1 (CHO-hPON1), which we had previously developed and described. Culture medium was harvested and analyzed for enzyme activity (arylesterase [ARE]) and occasionally for PON1 protein mass (competitive ELISA). Lactonase activity was analyzed using a substrate recently developed. Studies were also performed using primary rat hepatocytes and HepG2 cells, the latter transiently transfected with the coding region of the human PON1 gene.

**Recombinant PON1**

Recombinant PON1 (rePON1) variant G3C918 was expressed in E Coli, purified via a His tag and stored in Tris/detergent buffer (Tris 50 mmol/L, pH 7.8; CaCl2, 20 mmol/L; Tergitol, 0.05% [v/v]).

For more details of Methods, please refer to the online supplement at http://atvb.ahajournals.org.

**Results**

**Effect of Oxidation on HDL Composition and Structure**

Oxidation of HDL with AAPH to the level used in the present studies did not lead to aggregation of HDL peptides (supplemental Figure IA). In contrast, hypochlorite treatment caused the disappearance of monomeric size apo AI (supplemental Figure IB) and appearance of higher molecular weight bands, probably attributable to apo AI aggregation induced by chlorination. Such bands appeared at 100 μmol/L hypochlorite. Both AAPH and hypochlorite treatment induced changes to the electrophoretic mobility of HDL on agarose gels (supplemental Figure IIA and IIB), although in the case of hypochlorite there was no significant increase in the level of lipid peroxides. Neither did hypochlorite cause any significant changes in the phospholipid or cholesterol contents of HDL (data not shown) at least up to 0.2 mmol/L concentrations of the oxidant. Such observations have been previously reported for hypochlorite treatment of lipoproteins.

**PON1 Limits Oxidation of HDL in a Dose Dependent Manner**

The impact of variations in PON1 content of HDL on oxidation of the lipoprotein is shown in Figure 1. There was a decrease in the concentration of lipoperoxides that was dose-dependent on the presence of PON1. Lipoperoxides generated in HDL were negatively correlated with the activity of PON1 associated with the lipoprotein (r<-0.90 for all time points, P<0.01). In addition, the rate of accumulation of lipoperoxides with time was reduced in HDL preparations with higher concentrations of PON1.

**Oxidation of HDL Lowers PON1 Activity Released Into Conditioned Medium**

HDL has a stimulatory effect on secretion of PON1 into culture medium. When HDL, oxidized either with AAPH or copper ions, was incubated with CHO-hPON1 cells, there was a significant reduction in PON1 ARE activity of conditioned medium (P<0.001) compared with nonoxidized HDL (Figure 2A). The reduction of PON1 activity in the culture medium was dose-dependent on the degree of oxidation of HDL as shown in Figure 2B; all activity levels were P<0.01 compared with HDLco (0 LPO concentration). Our previous studies revealed that HDL-stimulated release of PON1 was saturable at 10 to 20 μg/mL of HDL protein, corresponding to the concentrations used in Figure 2A and 2B. Concentrations of HDL in the extra vascular compartment bathing cells and tissues are higher. Figure 2C shows that even with concentrations up to 300 μg/mL, oxidized HDL demonstrated a significantly lower capacity to increase PON1 activity in culture medium. Comparable results were obtained over short (3 hours) and long (20 hours) periods of incubation of HDL with the cells. The relevance of these observations to PON1 release from hepatocytes was examined in cultures of HepG2 transiently transfected with human PON1 and primary rat hepatocytes. Although releasing a much lower amount of PON1, there remained significant differences in the capacity of nonoxidized and oxidized HDL to promote release of the enzyme (Figure 2D).

A possible explanation for the lower levels of PON1 activity in the presence of HDLox may be reduced mass of PON1. This was examined by ELISA quantification of PON1 released into medium in the presence of HDLco or HDLox. As shown in Figure 2E, there was a significant reduction of PON1 mass measured in HDLox medium (P<0.03), corresponding to the reduction in the activity of PON1 released into medium (P<0.001).

**Analysis of Affinity Constants for HDL Binding and PON1 Release**

Our previous studies had established that HDL promoted PON1 release in a high affinity, saturable manner. To examine differing affinities for HDLco and HDLox (oxidized with AAPH) as an explanation for reduction in PON1, enzyme activity was analyzed as a function of HDL concentration. As shown in Figure 3A, for equivalent HDL concen-
trations, PON1 activity released into conditioned medium was consistently lower with HDLox as the acceptor lipoprotein. However, when apparent affinity constants were calculated based on Lineweaver-Burke plots of released enzyme activity (Figure 3B), there were no significant differences for the two forms of the lipoprotein (HDLco, 2.69±1.1μg HDL protein/mL; HDLox, 1.13±0.31μg HDL protein/mL; n=3 for both; P=0.1). Comparable results were obtained when HDL oxidized with copper ions was analyzed (HDLox, 2.46±1.5 μg protein/mL, mean of 2 analyses).

Figure 2. A, PON1 activity (mean±SD, n=4) in CHO-hPON conditioned culture medium containing HDL (10 μg protein/mL) either non-oxidized (control) or oxidized (AAPH and Cu). Control ARE activity was 0.48U/mL. B, PON1 activity (mean±SD, n=4) of CHO-hPON conditioned culture medium containing HDL (20 μg protein/mL), as a function of HDL oxidation (shown on the x axis as concentration of LPO/mg HDL protein). C, PON1 activity (mean±SD, n=6) in CHO-PON1 conditioned medium containing control (nonoxidized) or oxidized HDL at 100 or 300 μg protein/mL. D, PON1 activity (ARE) in medium conditioned with HepG2 cells or rat hepatocytes. Results are expressed as ΔOD/mL medium±SD, (n=6, for HepG2 and n=9 for rat hepatocytes). *P<0.001; **P<0.035 both v HDLco. E, PON1 activity and mass measured in CHO-hPON1 conditioned medium containing control (nonoxidized) or oxidized HDL at 100 or 300 μg protein/mL. Results are expressed as a percentage of the relative activity (mean±SD, n=14) and mass (mean±SD, n=40) present in HDLco containing medium. HDLco ARE activity, 0.61±0.001U/mL; mass, 0.31±0.05 μg/mL.

Figure 3. A, Dose–response curves for PON1 activity in CHO-hPON1 conditioned medium as a function of HDL concentration. Each point is the mean±SD (n=3) and the graph is representative of 3 independent analyses. B, Lineweaver–Burke double reciprocal plot of data from A. The intercept on the x axis represents the reciprocal of the apparent affinity constant. C, Binding of 125I-HDL to CHO-hPON1 cells as a function of HDL concentration. Results are given as ngHDL bound/mg cell protein, and are the means of duplicate determinations. Specific binding curves are given (representing total binding–non specific binding). D, Lineweaver–Burke double reciprocal plot of data from C. The intercept on the x axis represents the reciprocal of the binding constant.
A second series of studies examined the global affinity of the HDL complex for the PON1 secreting cells. Preliminary studies established that iodination of HDL did not modify its capacity to promote PON1 release (data not shown). Binding curves for the association of [125I]-HDLco and [125I]-HDLox with CHO-hPON1 cells are given in Figure 3C, which is representative of 3 independent determinations with 2 different preparations of HDL. The affinity constant (Figure 3D; 15.5 ± 1.7 μg HDL protein/mL; n = 3) of HDLco was comparable to that reported in the literature.21,23 HDLox bound to the cells with a higher affinity (6.6 ± 1.9 μg HDL protein/mL, n = 3; P < 0.02).

**Influence of HDL on PON1 Activity**

Previous studies have established that the lipid and apolipoprotein components of HDL play distinct but complementary roles in promoting PON1 release.9,24,25 To determine whether the reduced release of PON1 could be attributed to the lipid component alone of HDL, a total lipid extract of the lipoprotein was made and used in secretion studies as protein-free vesicles. PON1 release into conditioned medium containing oxidized and nonoxidized total HDL lipid extracts is shown in Figure 4A (representative graph of 4 independent analyses). The dose-response curves were largely superimposable, suggesting that oxidation had not modified the release process mediated by HDL lipids. Of particular relevance are the equivalents levels of PON1 activity found with both nonoxidized and oxidized HDL lipids. The presence of oxidized lipids does not appear to have an inactivating effect on secreted PON1. The lipid extracts were, however, less efficient than the native HDL complex, which promoted release of PON1 into conditioned medium at lower concentration (Figure 4A).

The latter point was further analyzed in conjunction with the ability of HDL to improve PON1 activity.9,24,26 Serum-free HAMS medium was conditioned (16 hours) with CHO-hPON1 cells and harvested. HDLco and HDLox (50 μg/mL) were added to the conditioned medium and assayed immediately for activity and after incubation (3 hour, 37°C). At t0, equivalent activities were found in both incubation mixes, despite the presence of oxidized lipids with HDLox. After incubation there was a significant rise in activity in both preparations (Figure 4B; P < 0.001), but activity rose to a lesser extent with HDLox (+12%) than with HDLco (+42%, P < 0.001 v HDLco). Studies were repeated with preparations of recombinant PON1. HDL-free rePON1 preparations were diluted into HDLco or HDLox samples (50 μg/mL) and activities measured immediately and after incubation (3 hours, 37°C). Dilution of rePON1 into HDL caused an immediate increase in activity, which was equivalent for HDLco and HDLox (Figure 4C). Subsequent incubation was associated with a decrease in rePON1 activity in the absence of HDL, probably caused by dilution of the Tergitol detergent stabilizing purified rePON1. In contrast, there were further increases in activity (P < 0.01) after 3 hours for both forms of HDL, although to a lesser extent with HDLox (+9%) than with HDLco (+47%, P < 0.001 v HDLox).

A final analysis compared the influence of HDLco and HDLox on PON1 with respect to its lactonase activity. Lactonase activity in CHO-hPON1 conditioned medium was significantly lower when oxidized HDL at 50 μg/mL (-13.8% v HDLco, P < 0.001) or 300 μg/mL (-14.6%, P < 0.001) (supplemental Figure IIIA) was the acceptor complex. When HDL was added to serum-free medium previously conditioned with the same cell line, there was an increase in lactonase activity for both HDLco (+17.2 ± 8.8%) and HDLox (+48 ± 4.8%), the increase being significantly greater (P < 0.0001) for nonoxidized HDL (supplemental Figure IIIB).

**Impact of Hypochlorite Treatment on HDL-PON1 Association**

Hypochlorite is an oxidising agent produced by the activity of myeloperoxidase in vivo. Pretreatment of PON1-free HDL with hypochlorite (HDL-OCl) before incubation with CHO-
hPON1 did not cause a significant decrease in the level of enzyme activity secreted by CHO-hPON1 cells (Figure 5A). In addition, HDL-OCl showed similar kinetics to control HDL with respect to stimulation of PON1 release from cells (Figure 5B). Thus, hypochlorite treatment of HDL did not appear to influence its ability to promote PON1 release and bind the enzyme. However, when HDL isolated from serum and containing active PON1 was treated with hypochlorite, a different picture emerged. As shown in Figure 5C, there was a dose-dependent decrease in PON1 activity on treatment with hypochlorite, with highest concentrations causing complete inactivation of the enzyme. This was paralleled by a dose-dependent decrease in monomeric PON1 as analyzed by Western blotting (Figure 5C and 5D). Hypochlorite-induced changes to PON1 activity and peptide occurred at concentrations lower than those that led to apoAI aggregation (supplemental Figure IB).

**Discussion**

The present study provides a novel and intriguing example of the consequences of oxidation for HDL function. Lipid peroxidation compromises the ability of the lipoprotein to obtain its full complement of PON1 activity. There is thus the potential for a vicious circle, whereby oxidation of HDL hinders its ability to acquire one of its components, PON1, which is suggested to protect the lipoprotein from oxidation. This could exacerbate the effects of oxidation on other HDL functions. The capacity of HDL to promote cholesterol efflux is diminished1,27 (a facilitating role for PON1 in cholesterol efflux has recently been proposed28). In this context, oxidation of HDL influences ABCA1-mediated cholesterol transport.29 Oxidation of HDL may also impair SR-B1–mediated cholesterol metabolism.21 In contrast, hypochlorite-mediated oxidation, which primarily concerns the protein components of HDL, had little effect on the ability of the lipoprotein to promote PON1 release from cells. However, PON1 already associated with HDL was extremely sensitive to hypochlorite treatment, rapidly losing activity at concentrations that are attainable in vivo under conditions of inflammation.30

Numerous studies have shown an inverse correlation between the activity of HDL-associated PON1 and oxidation of the lipoprotein13,31,32 as confirmed in this study. PON1 deficiency can thus have pathophysiological consequences for HDL. Our data indicate that several factors may contribute to reduced PON1 activity in oxidized HDL. One is a reduced capacity to stimulate PON1 release from the cell. It does not result from altered affinity of HDLox for the cell in that binding affinities measured as a function of PON1 activity9 did not differ between HDLco and HDLox. The latter was confirmed by complementary studies with radiolabeled HDL to measure affinity of the HDL complex, where HDLox tended to have a higher affinity than HDLco for the cell surface. This has been previously reported for LDL binding.21 The reasons for the reduced ability to remove PON1 from the cell are not clear, although it does mirror the reduced capacity to efflux cholesterol to HDLox.5,27 Modifications to the peptide components of HDL do not appear to be a major determinant as suggested by the studies with HDL-OCl.

The second factor appears to be a reduced capacity of HDL to further activate enzyme activity (an increase in PON1 specific activity). A positive impact on PON1 activity is a feature of the native HDL complex and has been independently reported by several groups.9,24–26 The mechanism is presently unclear, although correct orientation of the active
site into an optimal configuration may be a feature. Irrespective of the mechanism, there is clear evidence in the present report from 2 distinct preparations of PON1 that HDLox is deficient in its ability to further activate the enzyme.

A third possibility is that, once associated with HDL, PON1 may be particularly susceptible to inhibition by hypochlorite. Hypochlorite, which in vivo is derived from myeloperoxidase activity, was used in the present study as it principally targets peptide components. We could find no strong indication of lipid oxidation in treated HDL, as noted previously. One notable feature, however, was the extreme sensitivity of PON1 activity to hypochlorite treatment, despite a large excess of other peptides within HDL. Inhibition of PON1 activity was observed at HOCl:HDL protein ratios much lower than those previously used to study the effects of the oxidant on HDL function. A major effect on an HDL function (reverse cholesterol transport) provoked by an apparently mild, hypochlorite treatment regime has previously been reported. Myeloperoxidase is suggested to be one of the principal oxidizing agents giving rise to dysfunctional HDL in vivo. It exerts its effect on HDL predominantly within the artery wall where any buffering, antioxidant capacity of serum protecting PON1 would be diminished. In this scenario, ambient serum activities of PON1 would not reflect the effective activity or efficiency of the enzyme within the artery wall.

Finally, reduced, secreted PON1 activity in the presence of HDLox could also arise from inactivation of the enzyme by lipid peroxides. Our data suggest that it is not a major cause of reduced PON1 associated with HDL in the present studies. Firstly, there was an accompanying reduction of PON1 protein. Secondly, addition of HDLox to presecreted or recombinant PON1 did not decrease activity. Finally, protein-free oxidized HDL lipids did not show a reduction in PON1 activity in conditioned medium in comparison to protein-free nonoxidized lipids.

Phenylacetate (arylesterase activity) and paraoxon (para-oxonase activity) are the two substrates most commonly used to monitor PON1 activity. The present report focused on phenylacetate hydrolytic activity as it was recently shown to be a more accurate marker for the antioxidant activity of PON1 than is paraoxon. However, the antioxidant activity has been most tightly linked to lactonase activity of PON1. To ensure that our observations also apply to the latter activity, it was analyzed using a recently developed substrate. HDL oxidation was shown to affect lactonase activity in the same manner as it affected arylesterase activity; a reduced capacity to promote secretion and to increase enzyme activity.

The present study was based largely on the use of a CHO-hPON1 cell model of PON1 secretion, which we validated in a previous report. Its notable advantage is to minimize potentially confounding influences that could arise from cosecretion of lipids and apoA1 by hepatocytes. Confirmation was thus sought with hepatocytes that HDLox had a reduced ability to promote PON1 release from this cell type. This was shown to be the case using the human hepatocyte cell line, HepG2 transiently transfected with human PON1, and primary hepatocytes obtained from rat.

In conclusion, we demonstrate several effects of HDL oxidation on the PON1 content and activity of the lipoprotein. The clinical consequences of the observations await further investigation. However, it has been established by a number of independent studies that states of increased oxidative stress (diabetes, cardiovascular disease, smoking) are associated with reduced serum PON1. It is suggested to reflect in part PON1 inactivation by lipid peroxides. The present study indicates that HDL oxidation has a more fundamental impact on PON1 metabolism. It may have multiple consequences, as other HDL functions can be compromised by oxidation. The data underline the pathophysiological implications of HDL oxidation, which merits further investigation in the context of HDL as a primary therapeutic target and growing awareness of dysfunctional HDL.

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Disclosures
None.

References


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Supplementary files

Methods

Isolation of HDL

HDL was isolated from plasma pools of donors (5-6 healthy, normolipidic males and females) by cumulative flotation ultracentrifugation (1). Isolated HDL was dialysed against phosphate buffered saline (PBS) and stored in EDTA (100µM). Prior to use, EDTA was removed by extensive dialysis against PBS. PON1 protein was eliminated from dialysed HDL by immunoaffinity chromatography, as described previously (2). As EDTA inactivates PON1, absence of PON1 protein was checked by Western immunoblotting (2, 3).

HDL was also isolated from serum to maintain PON1 activity. Aliquots of these HDL were also subjected to immunoaffinity chromatography to remove PON1. PON1-free HDL was mixed with PON1-containing HDL to give HDL preparations with equivalent concentrations of lipids and protein but varying concentrations of active PON1.

Isolated HDL was also subjected to total lipid extraction and extracted lipids resuspended into protein-free vesicles, as described (4).

Oxidation of HDL

HDL (2mg protein/ml) was oxidised (37°C) under standardised conditions using 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH, 10mM; (5)). The reaction was stopped by placing the mixture on ice and AAPH removed by dialysis. Lipoperoxides (LPO) were determined using the Fox assay (6). Standardised oxidation conditions were established to give oxidised HDL (HDLox) containing 50-60nmoles LPO/mg HDL protein for the majority of studies reported herein, unless otherwise indicated. This corresponds to concentrations of HDL-associated LPO that have been reported in high risk populations (7, 8) and studies of cholesterol efflux (9). Copper sulphate (10µM final concentration) was occasionally used to oxidise HDL.
After addition of EDTA (100µM) to sequester copper ions, the HDL preparation was extensively dialysed against PBS.

Sodium hypochlorite treatment of HDL was performed as described (10). Briefly, HDL (1.0mg/ml) was incubated (2h, 37ºC in PBS) with various concentrations of sodium hypochlorite (Sigma-Aldrich, Buchs, Switzerland) diluted into PBS. The reaction was stopped with a 15x molar excess of methionine, and the HDL dialysed against PBS (4ºC). The treated HDL (HDL-OCl) was used within 24h of preparation.

Release of PON1 from cells

Studies of PON1 secretion were based on a Chinese hamster ovary (CHO) model stably transfected with human PON1 (CHO-hPON1), which we had previously developed and described (11). Briefly, CHO-hPON1 cells were seeded into 6-well culture plates (2x10^5 cells/well) and grown in complete medium for 2d. Cells were subsequently washed with 2x1.0ml HAMS F-12 nutrient medium (Sigma-Aldrich, Buchs, Switzerland) then incubated with serum-free HAMS supplemented with various concentrations of non-oxidised HDL (HDLco) and HDLox. The culture medium was harvested and analysed for enzyme activity (arylesterase (ARE)) using phenylacetate as substrate, (3)) and occasionally for PON1 protein mass (by competitive ELISA (3)). Lactonase activity was also analysed using a substrate recently developed (12) and kindly provided by Dr D. Tawfik. Briefly, using 96-well microtiter plates, PON1 samples were added to substrate (thiobutyl butyrolactone (TBBL, 0.2mM) in buffer (Tris, 50mM, pH 8.0, CaCl_2, 1mM, NaCl, 50mM) containing Ellman’s reagent (DTNB, 0.5mM) (total volume 200µl). The reaction was monitored over 5min at 405nm using a microplate reader (Molecular Devices, Bucher Biotec AG, Basle, Switzerland) in kinetic mode.
Studies were also performed using primary rat hepatocytes (13) and HepG2 cells. The latter were transiently transfected with the coding region of the human PON1 gene, as described previously (11).

Iodination of HDL and binding studies

HDL was iodinated using the Iodo-Beads iodination reagent (Perbio Science, Lausanne, Switzerland) according to the manufacturer’s instructions. Binding studies were performed as described (14) with non-oxidised and oxidised $^{125}$I-HDL. In the latter case, oxidation was performed both before and after iodination of HDL with comparable results. Briefly, CHO-hPON1 cells ($2 \times 10^5$ cells/well) were seeded into 6-well plates and grown in full medium for 2 days. Prior to use, cells were washed with 1x1.0ml serum-free HAMS medium then incubated (1h) with HAMS containing fatty acid-free BSA (0.5%w/v). Cells were washed with HAMS medium (1x1.0ml) then incubated (37°C, 1.5h) with various concentrations of $^{125}$I-HDL (control and oxidised) in HAMS + fatty acid-free BSA. Plates were subsequently chilled on ice (10min), washed rapidly (2x1.0ml) with ice-cold Tris-buffered saline (TBS; 50mM Tris, pH 7.4, 0.15M NaCl) containing BSA (2mg/ml), incubated 5min (on ice) with the same buffer (1.0ml) then washed rapidly (2x1.0ml) with ice-cold TBS. Finally, cells were dissolved in 1.4ml NaOH (0.1M) and aliquots counted for radioactivity and analysed for protein (15). Binding studies were performed in the absence (total binding) and presence (non-specific binding) of non-labelled HDL (1mg/ml) to give specific binding (total – non-specific).

Recombinant PON1

Recombinant PON1 (rePON1) variant G3C9 (derived from directed evolution of rabbit, human, mouse and rat PON1 genes (16) was expressed in E. Coli, purified via a His tag and stored in Tris/detergent buffer (Tris 50mM, pH 7.8; CaCl$_2$, 20mM; Tergitol, 0.05% (v/v)).
Statistical analyses

Statistical analyses were performed with the paired and unpaired Student’s t-test
References


**Supplemental figures**

Legends

Figure I: Silver stained SDSPAGE profiles of peptides from HDL A) non-treated (N) or AAPH oxidised (O; 62nmol LPO/mg HDL protein) and B) treated (1.0mg/ml HDL protein) with hypochlorite at indicated concentrations (mM).

Figures II: Agarose gel electrophoretic profiles of HDL A) non-oxidised (1, 2) or oxidised with AAPH (3, 62nmol LPO/mg HDL protein; 4, 148nmol LPO/mg HDL protein) and B) non-treated (1) or treated (2) with hypochlorite (0.2mM). Bands were stained with Coomassie Blue.

Figure III: A) Lactonase activity (mean±SD, n=8) in medium conditioned for 16h with HDLco or HDLlo at 50µg/ml (Exp 1) or 300µg/ml (Exp 2).

B) Lactonase activity (mean±SD, n=8) of CHO-hPON1 conditioned serum-free medium to which HDLco or HDLlo (50µg/ml) had been added, after removal from cells, and incubated 3h, 37°C.
Figure III

A

- HDLco
- HDLox

Exp 1
Exp 2

OD/min/ml

B

- HDL
+ HDLco
+ HDLcox

Complex

OD/min/ml