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Original Contribution

HDL-associated paraoxonase-1 can redistribute to cell membranes and influence sensitivity to oxidative stress

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ABSTRACT

Paraoxonase-1 (PON1) is a high-density lipoprotein (HDL)-associated serum enzyme thought to make a major contribution to the antioxidant capacity of the lipoprotein. In previous studies, we proposed that HDL promoted PON1 secretion by transfer of the enzyme from its plasma membrane location to HDL transiently anchored to the hepatocyte. This study examined whether PON1 can be transferred into cell membranes and retain its enzymatic activities and functions. Using Chinese hamster ovary and human endothelial cells, we found that recombinant PON1 as well as PON1 associated with purified human HDL was freely exchanged between the external medium and the cell membranes. Transferred PON1 was located in the external face of the plasma membrane of the cells in an enzymatically active form. The transfer of PON1 led to a gain of function by the target cells, as revealed by significantly reduced susceptibility to oxidative stress and significantly increased ability to neutralize the bacterial virulence agent 3-oxo-C_{12}-homoserine lactone. The data demonstrate that PON1 is not a fixed component of HDL and suggest that the enzyme could also exert its protective functions outside the lipoprotein environment. The observations may be of relevance to tissues exposed to oxidative stress and/or bacterial infection.

A number of mechanisms have been invoked to explain the capacity of high-density lipoprotein (HDL) to reduce the risk of vascular disease. Facilitated cholesterol excretion involving reverse cholesterol transport predominates, with persuasive evidence for complementary, risk-reducing anti-inflammatory, antioxidant, and antithrombotic functions of the lipoprotein [1]. Another feature of HDL function that contributes to the protective capacity is its ability to redistribute molecules, both lipid and protein, in nature. Reverse cholesterol transport is the principal example, with cholesterol being removed from cells and deposited in the liver, without uptake of the entire HDL complex, as is the case with low-density lipoprotein (LDL) [2]. Apolipoproteins (apo) C and E are suggested to shuttle between HDL and chylomicrons/very low density lipoproteins (VLDL) to facilitate lipolysis and removal of these triglyceride-rich lipoproteins [3]. Both processes are considered antiatherogenic.

Paraoxonase-1 (PON1) has attracted attention as an HDL-associated enzyme with an antioxidant function [4]. Studies of animal models [5–7] and in humans [8,9] are consistent with the hypothesis that PON1 decreases the risk of cardiovascular disease by lowering levels of oxidative stress, notably lipoprotein oxidation. In earlier studies, we proposed that HDL acquires PON1 by desorption from the hepatic cell membrane in a process probably mediated or facilitated by transient anchoring of HDL to the membrane [10]. This implies transfer of PON1 from the cell membrane to the cell membrane-like outer shell of the lipoprotein. In this study we have examined the possibility that the transfer may also occur in the reverse direction, from HDL to cell membranes. We also examined whether some of the functions attributed to PON1 were then associated with the recipient cell. Such proof of principal would demonstrate the ability of PON1 to operate outside its HDL transport vector and indicate a novel protective function of HDL.

Methods

Preparation of recombinant PON1 and HDL

Recombinant PON1 (rePON1) was prepared as described previously [10]. It was stored in Tris buffer (Tris 50 mM, pH 7.8, $CaCl_2$ 20 mM containing Tergitol, 0.05% (v/v)) and diluted 500- to 1000-fold into cell culture medium. Human HDL (δ 1.063–1.21 g/ml) was isolated from serum samples by density gradient ultracentrifugation [11], dialyzed against Tris buffer, and sterilely filtered for storage at 4 °C.

Abbreviations: HDL, high-density lipoprotein; PON1, paraoxonase-1; rePON1, recombinant PON1; VLDL, very low density lipoprotein; LDL, low-density lipoprotein; CHO, Chinese hamster ovary; HUVEC, human umbilical vein endothelial cell; AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; oxLDL, oxidized low-density lipoprotein; HSL, homoserine lactone; DCFH-DA, dichlorofluorescein diacetate; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone.

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Enzyme activity assays

PON1 enzyme activity was analyzed as phenylacetate hydrolysis (arylesterase activity [12]). Cell-associated activity was measured as described previously [10]. Briefly, cells were harvested, resuspended, and incubated with phenylacetate for 5 min before being centrifuged. The OD_{270} of the supernatant was measured to determine the extent of phenylacetate hydrolysis.

Cell culture

Three cell types were employed for the studies. Chinese hamster ovary (CHO) cells were maintained in culture as described previously [10]. To establish the potential physiological relevance of the observations, complementary studies were undertaken with endothelial cells. Primary cultures of human umbilical vein endothelial cells (HUVECs) were isolated as described [13] and cultured in EGM-2 medium (Cambrex, East Rutherford, NJ, USA). The endothelial cell line EA.hy926, generously provided by Dr. S. Horke, was cultured as reported previously [14]. Unless otherwise stated cells were preloaded by incubating with rePON1 (10 μg/ml) or human HDL containing PON1 (HDL–PON1, 1.0 mg/ml protein) for 15–16 h in serum-free culture medium. Preliminary studies established that this was sufficient to establish an equilibrium between membrane-bound and culture medium PON1.

Analysis of cell cultures by immunofluorescence was performed as described previously [10].

PON1 transfer to cells

Transfer of PON1 to cells was also analyzed immunochemically by Western blotting. After transfer, cells were washed (phosphate-buffered saline (PBS), 0.1% (w/v) bovine serum albumin) to remove absorbed material and then dissolved in lysis buffer (50 μl; Tris–HCl 50 mM, pH 7.4; NaCl 150 mM; glycerol 10% (v/v); EDTA 2 mM; EGTA 2 mM; Triton X-100 1% (v/v); β-glycerophosphate 40 mM; NaF 50 mM). Aliquots of lysed cells were fractionated by SDS–PAGE [10] and immunoprobed with anti-PON1 antibodies. Staining intensities were determined with the Odyssey infrared imager (Li-Cor Biosciences, Bad Homburg, Germany), which contains an integrated system for analyzing band intensity.

Determination of homoserine lactonase activity

Cells, preloaded with rePON1 or human HDL–PON1, were incubated with 3-oxo-C_{12}-homoserine lactone (3-oxo-C_{12}-HSL; 1.0 μM, 2 h, 37 °C) in Hanks' balanced salt solution (HBSS). After incubation the quantity of 3-oxo-C_{12}-HSL remaining in the medium was measured in a β-galactosidase-based bioassay using *Escherichia coli* strain MG4λI $_1$4 (pPCS1; a generous gift from Professor C. van Delden) as described previously [15].

Proteinase K treatment

Proteinase K treatment was carried out as described by Horke et al. [16]. Briefly cells were cultured in six-well plates and treated with proteinase K (25 μg/ml, 37 °C, 15 min) in PBS before addition of phenylmethylsulfonyl fluoride (5 mM) and lysis buffer. The cell extracts were then analyzed by Western blotting for the presence of PON1.

Downregulation of scavenger receptor BI (SR-BI) expression

SR-BI in CHO cells was downregulated using small interfering RNA (siRNA). The siRNA sequences were established using the Invitrogen BLOCK-iT designer and are given in Supplementary Table 1.

Protein expression was analyzed in whole-cell extracts by Western blotting using antibodies specific for SR-B1 (Novus Biologicals, Littleton, CO, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore, Zug, Switzerland) and the relevant second antibody.

Preparation of oxidized LDL

LDL (2 mg/ml LDL protein) was oxidized (6 h, room temperature, in the dark) using 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH; 50 mM) to give oxidized LDL (oxLDL) containing 40–60 nmol lipoperoxide/mg LDL protein as measured by the FOX assay [17].

Detection of reactive oxygen species

Reactive oxygen species (ROS) production in cells was monitored using dichlorofluorescein diacetate (DCFH-DA). DCFH-DA crosses the cell membrane and is converted enzymatically to nonfluorescent dichlorofluorescein. DCFH is oxidized in the presence of ROS to fluorescent dichlorofluorescein [18].

CHO cells were plated in six-well plates (8×10^5 cells/well) and preloaded with rePON1 overnight before incubation (2–4 h) with AAPH (25 mM) or oxLDL (0.1 mg/ml) in Hams F-12 cell culture medium. They were subsequently rinsed, trypsinized, and incubated with DCFH-DA in PBS (10 μM, 25 min, 37 °C in the dark). Fluorescence was analyzed using a FACSscan (Becton–Dickinson) flow cytometer recording green fluorescence with a 530-nm filter. Analyses were performed on 10,000 events.

Alternatively, CHO or EA.hy926 cells were plated in 96-well plates (8×10^4 cells/well) and preincubated overnight with rePON1 or HDL–PON1. The following day they were washed and incubated with the luminol derivative L-012 (Wako Chemicals, Neuss, Germany) in HBSS (0.5 mM, 37 °C, 15 min) before the addition of AAPH (25 mM) or DMNQ (2,3-dimethoxy-1,4-naphthoquinone; 10 μM; Alexis Bio, Switzerland) in Hams F-12 cell culture medium. ROS-induced chemiluminescence was determined every 15 min for 2 h.

Immunohistochemical analysis of PON1

Experimental intimal thickening was induced in the thoracic aorta of adult male Wistar rats by removal of the endothelium using an inflated embolectomy catheter as previously described [19]. Immunohistochemistry was performed on 5-mm-thick aortic sections [20,21] (untreated, $n=3$; treated, $n=3$, sacrificed 15 days after treatment). Before the first antibody was used, immunoreactivity was intensified by 1× microwave treatment (750 W, 5 min in citrate buffer 10 mM, pH 6.0) for anti-smooth muscle actin antibody or 1× pressure cooker treatment (3 min, citrate buffer) for anti-PON1 antibody. Goat anti-mouse biotinylated antibodies (Dako, Glostrup, Denmark) were used as secondary antibodies and the presence of the specific proteins was revealed by means of streptavidin–biotin–peroxidase (Dako) followed by hemalun counterstaining. Images were acquired with an Axioskop 2 (Carl Zeiss) using a 3200 K tungsten light with a high-sensitivity, high-resolution CCD color camera (Axiocam; Carl Zeiss) and plan-neofluar 10×/0.3 and 40×/1.3 objectives (Carl Zeiss).

Cyclodextrin treatment of cells

The cholesterol content of CHO cell membranes was modulated by preincubation of cells with cyclodextrin (5.0 mM, 2 h) [22]. Cells were then analyzed for transfer of rePON1 by Western blotting, as described above. Treated and untreated cells were extracted with chloroform:methanol (2:1 v/v) and dried aliquots analyzed for cholesterol using a commercially available colorimetric kit (Randox, Antrim, UK).

Results

PON1 can transfer into the cell membrane

Recombinant PON1 added to culture medium associated with CHO cells and HUVECs over 15 h of incubation (Fig. 1A). Whole-cell extracts showed PON1 to be present in a nondegraded form (MW 43 kDa) with no indication of a smaller molecular weight (23 kDa) degradation product of the enzyme that can be detected with the same antibody. Parallel studies with human HDL containing active PON1 also showed transfer of the enzyme to the cells, as it could be detected in whole-cell extracts in a nondegraded form (Fig. 1A). No apoAI could be detected in the extracts, suggesting the presence of PON1 in the absence of apoAI/HDL. To confirm the presence of PON1 in the cell membrane, enzyme activity was determined in non-permeabilized cells. After preincubation with rePON1 (Fig. 1B), arylesterase enzyme activity was detected in the cell membrane of CHO and EA.hy926 cells, indicating the presence of active PON1. Studies of HUVECs and EA.hy926 cells using human HDL gave comparable results, with enzymatically active PON1 detected in nonpermeabilized cells (Fig. 1B). Confirmation of the cell membrane location of PON1 was furnished by immunofluorescence, which revealed PON1 protein on the nonpermeabilized surface of cells preincubated with rePON1 (Fig. 1C). Final confirmation of the cell membrane location of transferred PON1 was demonstrated by incubation of nonpermeabilized cells with the proteolytic enzyme proteinase K. As shown in Fig. 1D, whole-cell extracts of proteinase K-treated cells revealed a complete loss of the PON1 signal in immunoblots. This strongly suggests that nondegraded PON1 detected in whole-cell extracts after preincubation with recombinant enzyme or HDL is located at the external surface of the cell membrane.

PON1 can shuttle between the cell membrane and HDL

Based on preceding studies we proposed that HDL acquires PON1 by desorption from a plasma membrane location in cells synthesizing the enzyme [10], as also suggested by others [23]. Thus we examined whether PON1 transferred into the cell membrane could relocate to HDL. After preincubation of cells with rePON1 or HDL–PON1, the medium was removed and cells were incubated with complete medium containing 5% (v/v) fetal calf serum. Fig. 2A shows that the enzyme, associated with CHO cells and HUVECs after preincubation with rePON1 (time 0), is lost from the cells during subsequent incubation with complete medium. This is accompanied by the appearance of PON1 protein and enzyme activity in the culture medium (Figs. 2 A and B). Complementary studies with human HDL as the source of PON1 gave comparable results, as illustrated for HUVECs (Fig. 2C). Human PON1, present in HUVECs after the preincubation period (time 0), was gradually lost from the cells during subsequent incubation with complete medium. Conversely, PON1 protein was absent from the culture medium at time 0 and appeared during subsequent incubation (Fig. 2C). The human PON1 protein signal was lost from the culture medium with time, which seems to reflect the lower stability of human compared to recombinant PON1 [24].

SR-BI, cyclodextrin, and PON1 transfer

We analyzed a possible role for the HDL receptor SR-BI in the transfer of PON1 to the cell membrane. CHO cells were treated with SR-BI siRNA to lower expression of the receptor and then analyzed for transfer of rePON1. SR-BI siRNA treatment reduced protein expression to $34.0 \pm 8.0\%$ ($n = 3$) of that in scrambled-RNA-treated cells

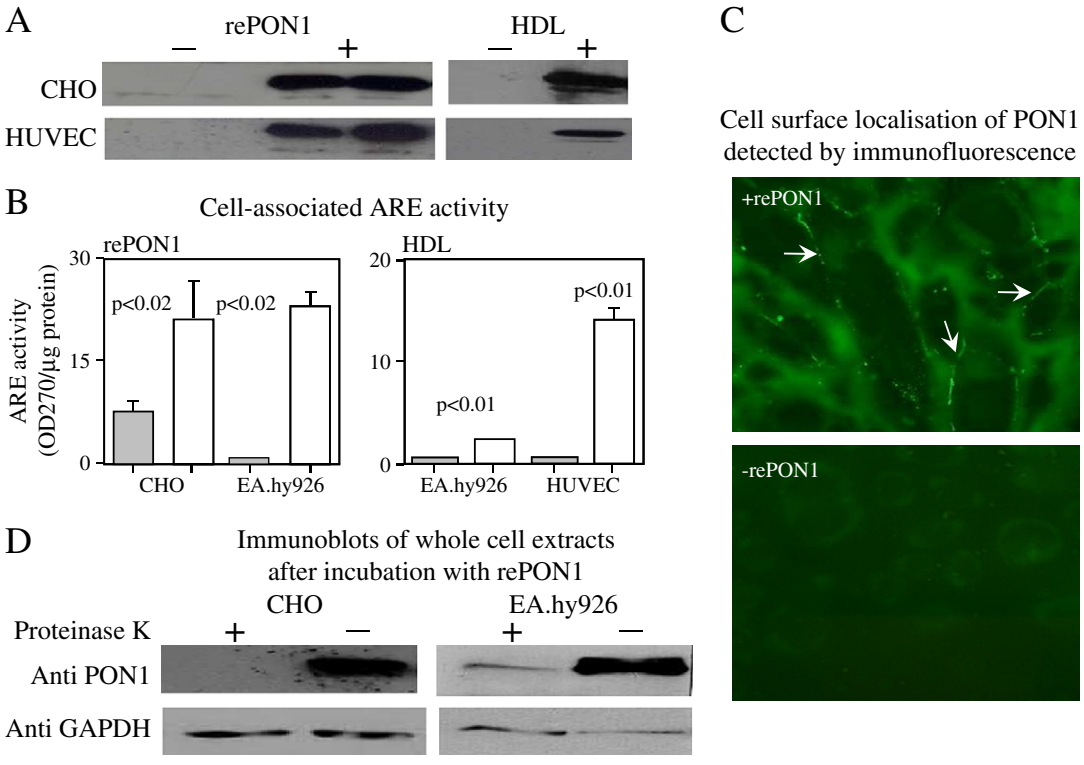

Fig. 1. Cell membrane association of PON1. (A) Immunoblot with anti-human PON1 of whole-cell extracts of CHO cells or HUVECs after incubation without or with rePON1 or human HDL. (B). Cell-associated PON1 enzyme activity (arylesterase; ARE) after incubation of CHO cells, HUVECs, or EA.hy926 cells without (shaded bars) or with (open bars) rePON1 or human HDL. *p* values are for comparison of activity between cells ± rePON1 or HDL. (C) Immunofluorescent localization of human PON1 to CHO cells after preincubation with (+ rePON1) or without (− rePON1) PON1. (D) After preincubation with rePON1, CHO or EA.hy926 cells were treated or not with proteinase K and then whole-cell extracts were subjected to immunoblotting with anti-human PON1 or GAPDH.

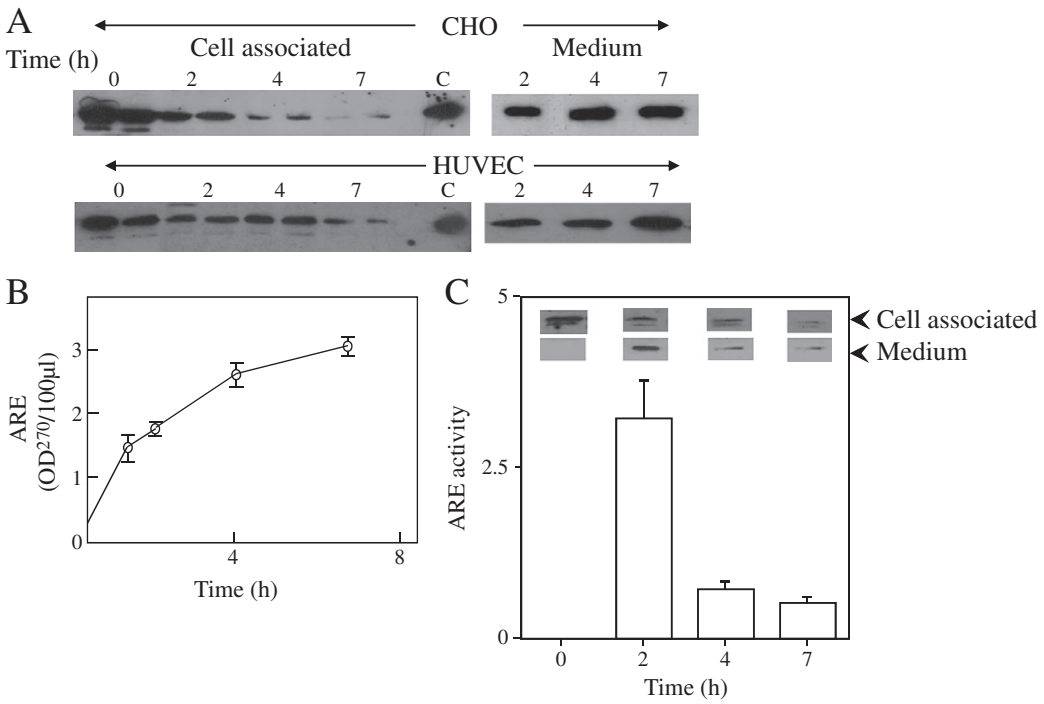

Fig. 2. Cell-associated and culture medium-contained PON1 after transfer of PON1-preconditioned cells into PON1-free medium. (A) Immunoblot with anti-human PON1 of total cell extract and conditioned culture medium from CHO cells and HUVECs preloaded with rePON1. (B) Paraoxonase activity (ARE) released from CHO cells into PON1-free medium. (C) HUVECs were preincubated with human HDL containing PON1, before incubation in PON1-free medium. Immunoblots of PON1 peptide in whole-cell extracts and culture medium up to 7 h after transfer to PON1-free medium. The graph shows paraoxonase activity (ARE) released into culture medium.

(Supplementary Fig. S1). When transfer of rePON1 into the CHO cell membrane was analyzed, PON1 associated with the SR-BI siRNA-treated cells was also significantly reduced ($p<0.05$), to 39.6 ± 6.0% of that observed in scramble-treated cells. Parallel studies with HDL showed a 72.1% reduction in SR-BI expression and a 60.5% reduction in transfer of PON1 (Supplementary Fig. S1).

Treatment of CHO cells with cyclodextrin lowered total cholesterol content by 47.0% (180.0 ± 5.0 vs 85.0 ± 6.0 µg cholesterol/mg cell

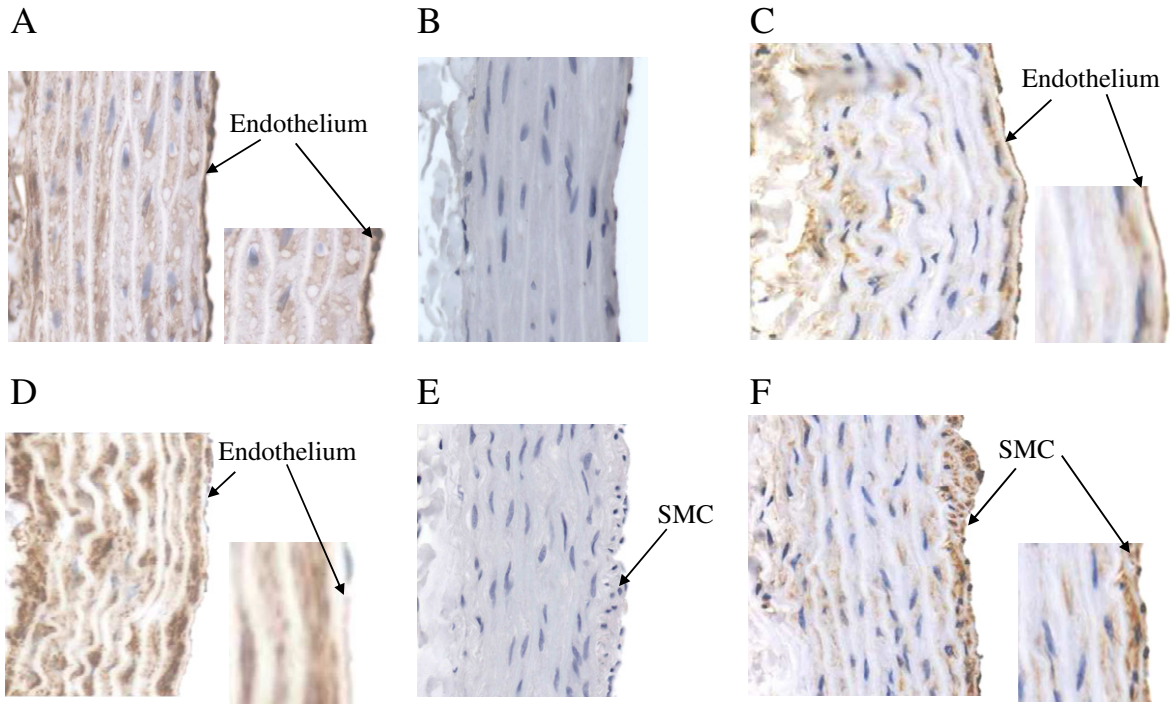

Fig. 3. Immunohistochemical analyses of PON1 in rat arterial tissues. Arterial tissue samples from control (untreated) and balloonized rats (15 days after treatment) immunoprobed with (A, C, F) anti-PON1, (D) anti-actin, or (B, E) a negative control (no primary antibody). (A) Section from control, untreated aorta; (B) negative control of untreated aorta; (C) aorta section from 15-day-treated rat that had retained an endothelial layer; (D) aorta section from 15-day-treated rat without endothelium; (E) negative control for treated rat; (F) aorta section from 15-day-treated rat. Insets show magnified views of primary image. SMC, smooth muscle cells in developing, intimal thickening.

protein). PON1 transfer into cells was increased (122.4 ± 6.0%, $p<0.05$, $n=3$, Supplementary Fig. S2).

PON1 in rat arterial tissues

The presence of PON1 was analyzed in rat arterial tissues as an example of a tissue particularly exposed to the circulation, exploiting the cross-reactivity of the monoclonal antibody with rat PON1. Fig. 3A shows immunohistochemical analysis of PON1 in healthy, nonstressed intact arterial sections from untreated rats. It reveals marked staining of the endothelial cell layer with minor staining of the underlying cell layers of the intima. A second series of rats was subjected to a balloonization procedure to remove the endothelium, which leads to development of experimental plaques [20,21]. Fig. 3C shows a section in which the endothelium remained intact. Again, there is marked PON1-specific staining of this endothelial layer, with little staining of the underlying smooth muscle cells. In contrast, Fig. 3F shows the smooth muscle cells exposed to the circulation because of complete removal of the endothelium. There is marked PON1 labeling of the surface cells. Studies with an anti-apolipoprotein AI antibody showed negligible staining of tissues (Supplementary Fig. S3).

Functional properties of PON1 transferred to cells

Oxidative stress

That PON1 transferred to the cell membrane was enzymatically active suggested that it may manifest other functional properties. This possibility was analyzed using isolated, purified rePON1 to eliminate the potentially confounding effects of the HDL complex, as the lipoprotein can beneficially influence cell metabolism and response to oxidative stress [25].

To examine the impact on oxidative stress, CHO cells containing transferred rePON1 were incubated either with AAPH, to generate free radicals, or with oxLDL. The impact on cell oxidative stress was monitored by FACS analysis of DCFH-loaded cells. As shown in Figs. 4 A and B, both AAPH and oxLDL induced a significant degree ($p<0.01$) of oxidative stress within the PON1-free cells, the effect being more pronounced for AAPH. Loading the cells with PON1 by preincubation greatly reduced the level of cellular oxidative stress ($p<0.01$) during subsequent incubation with AAPH or oxLDL.

A second series of studies analyzed oxidative stress induced in EA. hy926 cells by treatment with AAPH. As indicated by L-012 monitoring of oxidation-induced luminescence, there was a gradual rise in the level of intracellular stress in PON1-free cells (Fig. 5A), which peaked and subsequently receded. PON1-loaded cells reacted in a qualitatively similar manner (Fig. 5A), with a parallel, gradual rise in intracellular stress. However, the level of oxidative stress was significantly lower ($p<0.01$, $n=7$) than that observed in PON1-free cells. For comparison, the responses of CHO cells to the same experimental conditions are shown in Fig. 5B, with the difference between PON1-containing and PON1-free cells also being significant ($p<0.01$, $n=4$).

The impact of transferred PON1 on intracellular sources of oxidative stress was also analyzed using DMNQ, which produces ROS intracellularly after absorption and activation. DMNQ treatment of EA.hy926 cells provoked a rise in intracellular oxidative stress (Fig. 5C) that was significantly lower ($p<0.05$) if the cells were preloaded with PON1 (Fig. 5C).

Homoserine lactonase activity

Recent studies [26,27] have established that PON1 also expresses a lactonase activity, which allows neutralization of homoserine lactones (HSL). The lactonase activity of membrane-transferred PON1 was

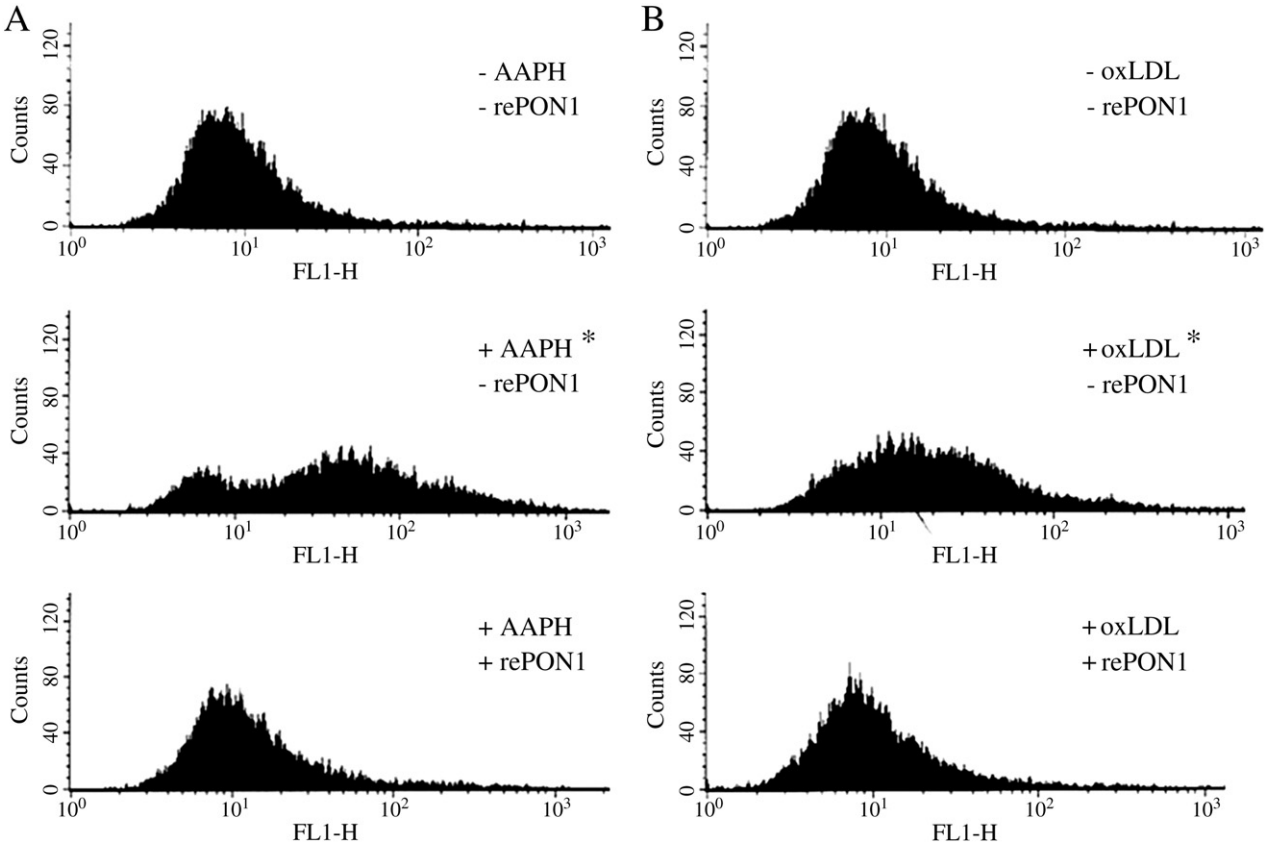

Fig. 4. FACS analysis of oxidative stress induced in CHO cells preloaded or not with rePON1 and treated exogenously with (A) AAPH or (B) oxidized LDL (oxLDL). One of three independent analyses; *$p<0.01$ vs the two other graphs in (A) or (B).

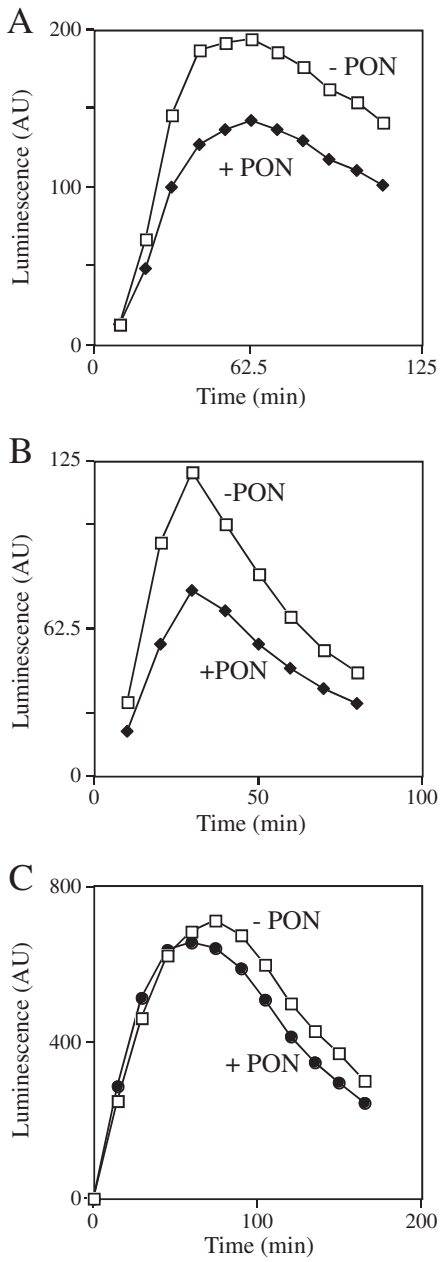

Fig. 5. (A, B) Monitoring by L-012 of intracellular oxidative stress induced by exogenous AAPH in EA.hy926 (A) or CHO (B) cells treated or not with rePON1. (C) Effect of added rePON1 on oxidative stress induced intracellularly by DMNQ and monitored with luminol.

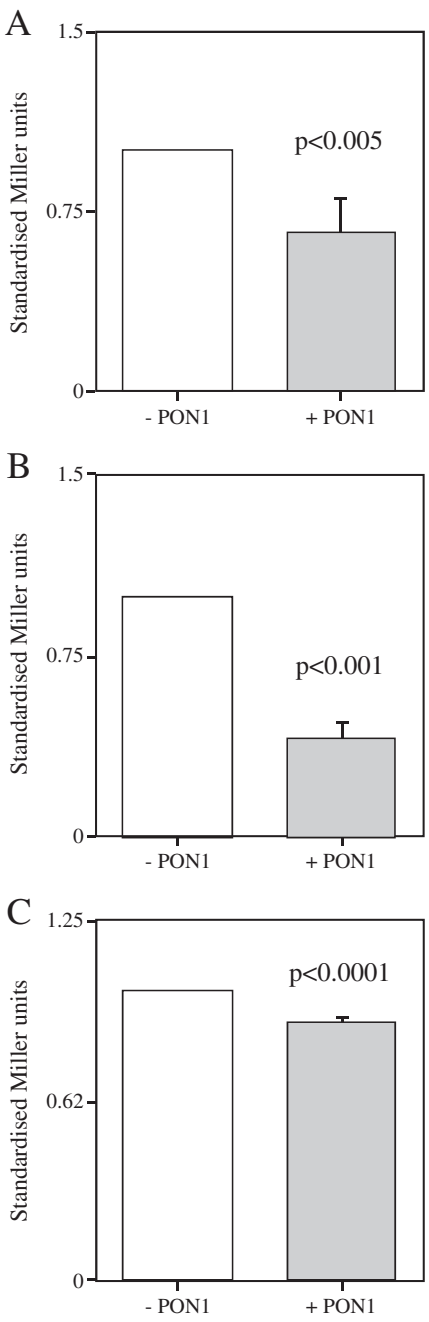

Fig. 6. HSL-neutralizing capacity of (A, C) CHO cells or (B) EA.hy926 cells loaded or not with rePON1 (A, B) or HDL (C). After incubation of HSL-containing medium with the cells, the medium was recovered and the remaining HSL analyzed in a β-galactosidase-based bioassay using *E. coli* MG4λI $_1$4 (pPCS1).

analyzed using CHO and EA.hy926 cells. When rePON1 was added to the culture medium, the cells acquired the capacity to hydrolyze HSL, as clearly illustrated in Figs. 6 A and B. This lactonase activity was also observed in CHO cells to which PON1 was transferred from isolated human HDL (Fig. 6C).

Discussion

HDL accomplishes a number of functions that can beneficially influence the atherosclerotic process. A notable feature of HDL metabolism that differentiates this lipoprotein subclass from LDL and VLDL is the ability to transfer and/or exchange lipids and peptides with cells and with other lipoproteins. This is exemplified by cholesterol, which can be removed or transferred to cells via the agencies of ABC transporters and SR-BI, respectively [2], and also transferred from HDL to LDL and VLDL [2]. The process is well established as being atheroprotective, as is the exchange of apolipoproteins C and E between HDL and VLDL or chylomicrons. Interestingly we demonstrated recently that PON1 can also be transferred between HDL and VLDL [28].

We suggest that exchange/transfer processes may be more extensive than these examples and could constitute another general mechanism by which HDL is cardioprotective. In this context, the aims of this study were twofold. One was to determine if our previously documented transfer of PON1 from the cell membrane to HDL was unidirectional. Clearly this is not the case. Whether originating from purified rePON1 or by transfer from HDL, PON1 was incorporated into the plasma membrane of cells that do not synthesize the enzyme.

Moreover, the exogenous PON1 could be rereleased into PON1-free medium. This was demonstrated not only for CHO cells, but also for the more physiologically relevant endothelial cell. Interestingly, cell-associated PON1 was entirely degraded by externally active protease. This confirmed its plasma membrane location. Our in vivo data are also consistent with transfer of PON1 to tissues that are particularly exposed to serum HDL. Endothelial cells in healthy vessels were found to contain PON1. The underlying smooth muscle cell layer showed little staining unless fully exposed to the circulation. In this case, extensive PON1 labeling became apparent particularly in the most exposed cells. This contrasts with the limited tissue distribution of PON1 mRNA [29]. Finally, our studies suggest that the HDL receptor, SR-BI, may influence the transfer process, as reduced SR-BI expression correlated with reduced transfer of PON1 into CHO cells. This is an intriguing observation that complements unpublished studies from our laboratory suggesting the SR-BI has a major role to play in the transfer of PON1 from cells to HDL. It may be linked to the need to prevent exposure to the aqueous milieu of the retained, highly hydrophobic signal peptide.

Depletion of membrane cholesterol with cyclodextrin occasioned an increase in transfer to the cell membrane. It suggests that the cell membrane lipid composition may influence the transfer process. Interestingly, we showed previously [10] that secretion of PON1 from cells to HDL was reduced when the cholesterol content of HDL was increased. Further studies should examine whether the phospholipid composition of the cell membrane influences PON1 transfer.

In its membrane-bound and rereleased forms, PON1 was enzymatically active. This allowed us to address our second objective, which was to determine if the cell derived any benefit from PON1 incorporation into the external face of the cell membrane. One of the major activities attributed to PON1 is an ability to reduce oxidative stress [30]. We investigated whether target cells modified their susceptibility to oxidative stress after incorporation of PON1 into their cell membranes. Studies employed purified rePON1 to avoid the use of HDL. The latter is known to influence the response of cells to oxidative stress independent of PON1 [25]. Using two different sources of extracellular-induced oxidative stress (AAPH and oxLDL), we demonstrated significantly reduced intracellular oxidative stress in CHO and endothelial cells with membrane-bound PON1. We also analyzed the effect of specifically increasing intracellular oxidative stress with the agent DMNQ. A somewhat different oxidative stress profile was observed (Fig. 5C). Initially intracellular oxidative stress increased in comparable manners in cells with and without PON1, suggesting no impact of PON1 in this early stage of ROS production. Thereafter, the wane in stress levels was significantly more rapid in the PON1-containing cells. One possible explanation for this observation, given that membrane-bound PON1 seems to be active extracellularly, is that it may reflect the influence of the enzyme on reactive oxygen species released into the medium that could subsequently have an impact on the cell. Seeding of extracellular complexes with ROS of intracellular origin is a well-described phenomenon, thought to be instrumental in oxidation of LDL [31]. In addition, studies by King et al. [32] demonstrated that ROS produced intracellularly by DMNQ treatment were released into the medium within 2 h of initiating treatment.

In a series of studies Efrat and Aviram [33] have shown that PON1 can be taken up by macrophages, in which it was also demonstrated to influence favorably the redox status of the cell [33]. The process seems to differ substantially from that described above in that PON1 is internalized and subject to degradation. It may reflect a pathway specific to macrophages, which have a strong capacity to absorb peptides, linked to their defense function. Taken with our data, this suggests that exogenous PON1 may be taken up by cells by different mechanisms.

More recently, PON1 has been shown to exhibit lactonase activity, which is suggested to have been the ancestral activity of the PON family [26,27]. This is an important function that may link paraoxonases to innate immunity, as lactonases can act as quorum quenching agents to limit bacterial virulence. It has been clearly demonstrated that the paraoxonases, by hydrolysis of HSL, limit infection and biofilm formation by *Pseudomonas aeruginosa* [34]. All three members of the paraoxonase gene family can neutralize HSL, with paraoxonase-2 being the most active [34]. However, it is an intracellular enzyme, and under conditions where diffusion of HSLs into cells may be rate limiting for their inactivation activity, PON1 assumes greater importance [35]. Thus, the quorum quenching activity of serum is essentially due to PON1 [34]. In this context, extracellularly located PON1 could serve as an initial line of defense against *P. aeruginosa* infection. Our data are consistent with this suggestion, as cells exhibited an increased HSL inactivation capacity after incorporation of PON1 into their plasma membranes.

These proof-of-principle studies indicate that incorporation of exogenous PON1 from HDL into cell membranes leads to a gain of function by the target cells with respect to resistance to oxidative stress and inactivation of bacterial virulence agents. The ability of HDL, compared to other lipoprotein subclasses, to circulate with relative ease in the extravascular compartment, thus facilitating its interaction with tissues and cells, may be of importance in this respect. The data demonstrate that PON1 is not a fixed component of HDL and that the enzyme could also exert its protective functions outside the lipoprotein environment. Interestingly, recent immunohistochemical analyses of PON1 [29,36] revealed a much wider tissue distribution of the peptide than expected from gene expression studies. Our studies suggest one mechanism that could contribute to this phenomenon, which may be of particular relevance to the reported presence of PON1 in the atherosclerotic plaque [37].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.freeradbiomed.2010.09.002.

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