Paraoxonase-1 (PON1) is a high density lipoprotein (HDL)-associated serum enzyme that protects low density lipoproteins from oxidative modifications. There is a relative lack of information on mechanisms implicated in PON1 release from cells. The present study focused on a model derived from stable transfection of CHO cells, to avoid co-secretion of apolipoprotein (apo) A-I and lipids, which could lead to formation of HDL-like complexes. Our results indicate that, in the absence of an appropriate acceptor, little PON1 is released. The results designate HDL as the predominant, physiological acceptor, whose efficiency is influenced by size and composition. Neither lipid-poor apoA-I or apoA-II nor low density lipoproteins could substitute for HDL. Protein-free phospholipid complexes promoted PON1 release. However, the presence of both apolipoprotein and phospholipid were necessary to promote release and stabilize the enzyme. Immunofluorescence studies demonstrated that PON1 was inserted into the external membrane of CHO cells, where it was enzymatically active. Accumulation of PON1 in the cell membrane was not influenced by the ability of the cell to co-secrete of apoA-I. Release appeared to involve desorption by HDL; human and reconstituted HDL promoted PON1 release in a saturable, high affinity manner (apparent affinity 1.59 ± 0.3 μg of HDL protein/ml). Studies with PON1-transfected hepatocytes (HuH-7) revealed comparable structural features with the peptide located in a punctate pattern at the external membrane and enzymatically active. We hypothesize that release of PON1 involves a docking process whereby HDL transiently associate with the cell membrane and remove the peptide from the external membrane. The secretory process may be of importance for assuring the correct lipoprotein destination of PON1 and thus its functional efficiency.

Paraoxonase-1 (PON1) is a high density lipoprotein (HDL)-associated serum enzyme that protects low density lipoproteins (LDL) from oxidative modifications. In vitro studies have demonstrated the capacity of PON1 to prevent LDL (and HDL) oxidation by a variety of pro-oxidant factors, including cell-induced LDL oxidation (1, 2). Complementary studies have shown that the anti-oxidant activity of PON1 prevents LDL from acquiring a number of pathological characteristics associated with the atherosclerotic process, notably monocyte mobilization and gene activation (3). The PON1 knockout mouse model confirmed that absence of serum PON1 activity increases the level of lipoprotein oxidation, renders LDL more susceptible to oxidation, decreases the anti-oxidant capacity of HDL and leads to more extensive atheroma formation (4, 5). In man, we first demonstrated that PON1 was an independent, genetic risk factor for coronary disease (6, 7). This has been confirmed independently (8–11), although not consistently (12, 13). In subsequent studies we have shown that PON1 promoter polymorphisms, which affect promoter activity and serum PON1 concentrations (14), are also independent risk factors for coronary disease (15). This is consistent with gene expression being a determinant of the influence of PON1 on vascular disease. Several in vitro studies have shown a direct correlation between variations in PON1 activity and the capacity to prevent LDL oxidation (1, 2, 16). These observations are corroborated by in vivo studies where serum PON1 levels correlate inversely with levels of lipoprotein oxidation (17) and lesion formation (4, 5, 18). Thus PON1 concentration is an important determinant of the anti-oxidant capacity of HDL.

There is a relative lack of information on factors that modulate serum PON1 concentrations. Two logical considerations are gene expression and release of the enzyme (by hepatocytes, PON1 being of hepatic origin). In studies of the PON1 gene, we identified promoter polymorphisms and showed that they accounted for some 25% of variations in serum PON1 (14). With respect to factors influencing PON1 release, or the release mechanism, presently little data is available. HDL would appear to be an important consideration. PON1 is entirely associated with HDL in human serum (19) and there is a positive, albeit weak correlation between serum concentrations of PON1 and several anti-oxidant capacity of HDL.
and HDL (19). Moreover, HDL is necessary to maintain normal PON1 serum levels, as we demonstrated recently using human HDL deficiency syndromes as a model (20). Thus, while HDL appears to be the serum transport vector for PON1, nothing is known about its contribution to peptide release. The latter mechanism is the focus of the present study. Based on these studies, we propose a model for PON1 release in which HDL functions as an acceptor, facilitating desorption of the enzyme from an external cell membrane location, and stabilizing the released enzyme. It may involve binding of HDL to the surface of producing cells.2

MATERIALS AND METHODS

Cell Transfection—Human PON1 was cloned from a liver biopsy sample as described (21). It has a glutamine residue at coding region polymorphic position 192 and leucine at coding region polymorphic position 55. Chinese hamster ovary cells (CHO; dihydrofolate reductase-deficient) were transfected with the cDNA corresponding to the coding region of the human PON1 gene under the SV40 constitutive promoter, and a stable line was isolated by methotrexate resistance. The CHO-hPON1 line synthesizes and releases PON1 peptide with those of human serum PON1 (21). The human hepatocyte line, HuH-7, was transiently transfected with hPON1 as described (21).

Purified Peptides, Lipoproteins, and Reconstituted Lipoproteins—Apolliproteins (apo) A-I and A-II were purified from delipidated human HDL (hHDL) by gel filtration chromatography (22). Purified, recombinant human clusterin (apoJ) was a gift from Dr. L. French (Department of Dermatology, University Hospital, Geneva). Human HDL (d 1.063–1.21 g/ml) was isolated from a pool of fasting human normolipemic serum by sequential gradient ultracentrifugation (23). To remove endogenous PON1, hHDL was passed through an affinity column containing monoclonal antibodies against human PON1, as described (19). LDL (d 1.019–1.052 g/ml) was isolated from serum by sequential gradient ultracentrifugation and washed once. Traces of contaminating HDL were removed by immunoaffinity chromatography on an anti-human apoA-I affinity column (22).

Reconstituted HDL (rHDL) were prepared as described (24). Discoidal HDL containing apoA-I (rHDLAI) or apoA-II (rHDLAII) were prepared by the cholate dialysis technique using a palmitoyloleophosphatidyl choline (POPC)/protein ratio (w/w) of 2.5:1.0. Cholesterol-containing rHDL were prepared using a POPC/cholesterol/protein ratio of 2.5:0.12:1.0. The size of mixtures and homogeneous particles was estimated by non-denaturing gradient gel electrophoresis (24), using the Pharmacia Phast System (Amersham Biosciences, Inc.). rHDLAI consisted of a major population of particles with a diameter of 9.6 nm. Homogeneous rHDLAI of 9.6 and 17.0 nm were isolated from a reaction mixture containing POPC/apoA-I (3.25:1.0, w/g) by gel filtration. rHDLAII contained a single population of particles (10.0 nm). POPC liposomes were prepared by the same procedure used for the rHDL, but omitting protein. Phospholipid and cholesterol contents of HDL were determined by enzymatic methods; proteins by the method of Lowry (25).

Studies of PON1 Release—To analyze release of the enzyme, stably transfected CHO-hPON1 cells were first grown to confluency in Iscove’s modified Dulbecco’s medium containing fetal calf serum (5%) while CHO-apoAI cells transiently transfected with PON1 were grown for 24 h after transfection in fetal calf serum (5%). Medium was removed, the cells were washed with phosphate-buffered saline (PBS; Ref. 22) and, unless otherwise indicated, serum-free medium (Hanks’ balanced salt solution (HBSS; Ref. 26) containing the acceptor under test was added. Cells were grown for the indicated period of time and the medium harvested. Conditioned medium was analyzed for PON1 enzyme activity and peptide mass.

Immunofluorescence—Cell-associated PON1 was visualized by immunofluorescence as described (27), with minor alterations. CHO-PON1 cells or transiently transfected hepatocytes were incubated at 4 °C for 30 min with monoclonal anti-PON1 antibody in immunofluorescence buffer (Dulbecco’s modified Eagle’s medium containing fetal calf serum (5%) and 0.1 M Hepes). Cells were washed three times with immunofluorescence buffer followed by two washes with PBS before fixing with paraformaldehyde (3% in PBS, room temperature, 30 min). The fixative was removed with PBS (four washes), and cells were treated with goat anti-mouse IgG antibody coupled to FITC (30 min, 4 °C). After four washes with PBS, cells were mounted in 90% glycerol containing p-phenylenediamine and observed under a Zeiss Axioskop microscope. In each case transfected and control cells were photographed and printed under identical conditions.

PON1 Activity and Peptide Mass—PON1 activity (arylesterase (ARE)) was measured in conditioned medium using phenylacetate as substrate (22) (CV 1–2%). Unless otherwise stated, activity is expressed as ΔOD405/min. When cell-associated activity was measured, cells were harvested, resuspended, and incubated with phenylacetate for 5 min before centrifuging (1 min) to remove cells. The OD405 of the supernatant was measured to determine the extent of phenylacetate hydrolysis. Cell viability was tested after activity assays with Trypan Blue. Studies of cell-associated PON1 activity were also performed with the substrate β-naphthylacetate, which forms an insoluble red product in the presence of Fast Blue (29). Stain was removed by thorough washing with HBSS, and cells were fixed in formaldehyde (3%, 30 min), washed in HBSS, and mounted in glycerol (90%) for photographic analysis. PON1 mass was quantified by competitive immunassay (CV 7–8%) (28).

Gel Filtration, SDS-PAGE—Conditioned culture medium was concentrated (70% w/v) by ultrafiltration (Amicon model 202). Activity assay showed demonstrated negligible loss of PON1 during the concentration step. Concentrated, conditioned culture medium was fractionated by FPLC (19). Eluant was collected (0.6-mI volumes) and tested for PON1 enzyme activity. The presence of PON1 peptide was analyzed by SDS-PAGE and Western blotting using monospecific anti-PON1 antibody (19). Single dimension SDS-PAGE was performed as described previously (22).

Statistical Analyses—Differences in activity and peptide mass between treatments were examined by paired and unpaired Student’s t test.

RESULTS

PON1 Release in the Presence of Lipid Complexes and HDL Apolliproteins—Studies were initially performed using serum-free CHO-S-SFM II medium (Invitrogen, Basel, Switzerland) as control medium. A low level of PON1 activity was observed when cells were incubated with medium alone. When hHDL or rHDL (containing either apoA-I or apoA-II) were added, there was a 2–3-fold increase in the level of PON1 activity in the culture medium (Fig. 1a) compared with control. In contrast, human LDL did not increase activity above that observed with medium alone (Fig. 1a). Protein-free phospholipid vesicles also increased PON1 activity of culture medium, but to a lesser extent than HDL (Fig. 1a). ApoA-I and apoJ (clusterin) have been found in complexes with human PON1 (19). However, when lipid-free forms of these peptides were added to CHO-hPON1 cells, there was a minor increase (apoA-I) or no increase (apoJ) in activity (Fig. 1a). Subsequent analyses showed CHO-S-SFM II to contain low levels of phospholipid. To confirm our results, studies were repeated with lipid-free HBSS. As illustrated in Fig. 1b, qualitatively similar results were obtained in that PON1 activity of conditioned medium increased in the order control medium (HBSS)<POPC<hHDL<rHDL. However, compared with HBSS, HDLs now showed a greater capacity to increase PON1 activity of conditioned medium (3.5–4.6-fold). HBSS was used in subsequent studies.

The increase in enzyme activity of the conditioned medium was closely paralleled by an increase in peptide mass. This is illustrated by Fig. 2, which shows a strong correlation between increased activity and mass (r2 = 0.99, p = 0.0001, excluding apoJ-I, irrespective of the type of complex added to the culture medium. It demonstrates that increased activity in conditioned medium is due, at least in part, to accumulation of PON1 peptide in culture medium, consistent with an increased release of PON1. An exception was apoJ-I (Fig. 2). The increase in activity was not accompanied by a comparable increase in

2 The studies were presented in part in abstract form by S. Deakin, I. Leviev, L. Calabresi, G. Franceschini, and R. W. James (2001) at the 72nd meeting of the European Atherosclerosis Society, Glasgow, Scotland.
peptide mass. It suggests an influence of apoA-I on peptide activity/stability rather than PON1 release.

Stabilization of Enzyme Activity—As HDLs and individualapolipoproteins had divergent effects on the PON1 activity of conditioned medium, we analyzed next whether PON1 stability, as defined by enzyme activity, was also influenced by the acceptor complex. To this end, culture medium containing lipid complexes or apolipoproteins was conditioned with CHO-hPON1 cells for 24 h then removed from the cells. The conditioned, cell-free medium was immediately assayed, and the assays repeated after incubating medium 24 h at 37 °C. Fig. 3a shows percent loss of activities between the two analyses, while Fig. 3b shows absolute loss of activities as well as total, initial activities. Human HDL and both forms of rHDL were able to maintain enzyme activity during the additional incubation period. In contrast, there was substantial loss of activity from medium that contained phospholipid vesicles alone as additive (mean 25%; p < 0.01 v HDL complexes). Delipidated apoA-I was also unable to maintain the activity of PON1 during the supplementary incubation period (Fig. 3). Addition of protease inhibitors did not modify the results (results not shown) suggesting that protein degradation did not contribute to loss of activity. A second aspect that was analyzed concerned the effect of HDL on presecreted PON1. The enzyme was released into serum-free medium, which was removed from the cells prior to addition of rHDLAI or POPC. After 2 h of incubation, we observed a 33.7 ± 3.1% (n = 9) increase in activity with rHDL, and a 14.2 ± 2.0% (n = 6) increase in activity with POPC vesicles. Thus increased specific activity may also be one pathway by which HDL influences PON1 function. While greater stability and/or specific activity may contribute to higher PON1 observed in the presence of HDLs, the principal effect of the lipoprotein complexes is to promote PON1 release by CHO cells.

PON1 Association with Lipid Complexes—As we recently reported (21), PON1 secreted from transfected cells into medium containing HDL can be removed by anti-apoA-I antibodies, demonstrating the formation of complexes with HDL (21). To further analyze the fraction size with which PON1 was associated, conditioned medium containing as acceptor PON1-free hHDL (Fig. 4a), POPC vesicles (Fig. 4b), or rHDL (Fig. 4c) was fractionated by FPLC. PON1 enzyme activity profiles are given, and it was confirmed by immunoblotting that activities paralleled elution of PON1 protein (Fig. 4d, is given as an example, which shows immunoblots for PON1 and apoA-I arising from the rHDLAI profile (Fig. 4c). The principal PON1 peak localized to the same region of each elution profile, irrespective of the acceptor complex present in the medium. This suggests that PON1 preferentially associates with complexes of a particular size. It is underlined by the rHDL profile (Fig. 4, C and D) where PON1 was not primarily associated with the major apoA-I-containing fractions.

**Fig. 1.** PON1 enzyme activity as a function of added acceptor. a, the percent increase in PON1 enzyme activity of culture medium as a function of the acceptor added to the medium (concentration, 5 μg/ml phospholipid for complexes (n = 12–21); 20 μg/ml for proteins alone (n = 3–15)). CHO-hPON1 were grown (16 h) in test medium (CHO-S-SFM II) containing the indicated acceptor before harvesting and testing medium. CM, culture medium alone. b, the increase in PON1 enzyme activity as a function of the acceptor (concentrations as for a) in HBSS culture medium. (HBSS, open squares, rHDLAI, open triangles; hHDL, open circles; POPC, open diamonds).

**Fig. 2.** The correlation between PON1 activity and PON1 mass in culture medium (serum-free CHO-S-SFM II, 16 h) containing different acceptors. The data are representative results from analyses of different acceptors.
Influence of HDL Size and Composition on PON1—Human and reconstituted HDL had different capacities to promote release of PON1 activity from cells (Fig. 1) suggesting that HDL composition and/or size may influence this function. The possibility was analyzed with rHDL of defined composition and size. First, cells were incubated with homogenous rHDLAI of 9.6 or 17 nm in diameter and the activity of conditioned medium was measured. Large diameter rHDLAI was more efficient (p < 0.001, n = 24) at increasing PON1 in culture medium than small diameter particles (Table I). The results agree with those from FPLC analyses, where PON1 was found to associate with a particular fraction size, suggesting in turn that HDL size is a determinant of PON1 release by cells. To compare possible effects of HDL composition, non-esterified cholesterol was incorporated into smaller reconstituted rHDLAI (9.6-nm diameter). When they were incubated with CHO-hPON1 cells, PON1 present in medium was significantly decreased compared with the same particles without free cholesterol (Table I, p < 0.001, n = 16).

**Table I**

| Particle       | ΔOD270/Hμg protein | p       |
|----------------|--------------------|---------|
| Large rHDL     | 0.854 ± 0.076      | <0.001  (v small rHDL) |
| Small rHDL     | 0.711 ± 0.048      |        |
| Small rHDL + cholesterol | 0.495 ± 0.092 | <0.001  (v small rHDL) |

Lipid Complexes Promote PON1 Secretion in a Saturable Manner—The release of PON1 from CHO-hPON1 cells was analyzed as a function of the concentration of acceptor added to the culture medium. The increase in PON1 activity in the culture medium was saturable for each type of complex that was tested, both human and reconstituted HDL. Results with hHDL are given as an example in Fig. 5. Fig. 5A illustrates that binding was saturable and, moreover, that the binding curve was unaffected by the time of incubation with the acceptor. That is, with time more PON1 accumulated in the culture medium, but the affinity constant (Fig. 5B) did not vary. Affin...
ity constants were estimated by the Lineweaver-Burk plot using enzyme activity as a marker for apparent binding activity. The analyses gave estimates for binding constants of hHDL (based on protein content) of 1.59 ± 0.3 µg/ml (n = 3).

**PON1 Is Present in the CHO Plasma Membrane**—The association of PON1 with the stably transfected CHO cells was analyzed by immunofluorescence, using monoclonal, anti-human PON1 (19). As illustrated in Fig. 6a, PON1 could be detected in non-permeabilized cells as a punctate border to the cell (Fig. 6a, inset). This is suggestive of its presence in the external face of the plasma membrane. Although one rationale for using CHO cells was to avoid possible confounding effects arising from co-secretion of apoA-I, one potential explanation for the accumulation of PON1 in the membrane is that the enzyme may block due, precisely, to absence of co-secreted apoA-I. To test this possibility, PON1 was transiently transfected into CHO cells that had been stably transfected with human apoA-I (the CHO-apoAI cell line was generously provided by Dr. J. Owen, Royal Free and University College Medical School, London). Fig. 6c shows that PON1 was also present in a punctate pattern in the non-permeabilized membrane of CHO-apoAI-PON1 cells. Western blots (Fig. 7, lane 3) confirmed the secretion of apoA-I by the cell line.

The orientation of PON1 toward the external milieu was confirmed by analyses of enzyme activity. Using a substrate whose hydrolysis product is insoluble, strong staining of the cell surface (but not cell-free areas) was observed when the substrate was added to CHO-hPON1-transfected cells (Fig. 6e). It contrasts with the absence of staining at the surface of non-transfected CHO cells (Fig. 6f). Arrows indicate cells that became permeabilized during processing, and these manifest extensive, uniform staining. They support the proposal that staining of other cells is due to external PON1 activity rather than leakage of the substrate into the cell and internal hydrolysis. Moreover, EDTA blocked production of the red stain product by CHO-hPON1 cells (Fig. 6g), consonant with the known calcium sensitivity of the enzyme. This is also consistent with the enzyme activity responsible for the red stain being limited to the exterior of the cell.

Activity measurements were performed with phenylacetate as substrate, which yields a soluble product. No measurable activity was found when non-transfected CHO cells were incubated with the substrate. In contrast, activity was observed when phenylacetate was added to CHO-hPON1 cells (Table II; p < 0.0001). This activity was blocked (>90%) when EDTA was added to the medium. No activity above background could be detected in culture medium conditioned with cells for 20 min. It demonstrates that activity measured in the presence of CHO-hPON1 cells was not due to secreted enzyme.

**Fig. 5.** PON1 activity in culture medium as a function of the HDL concentration. A, a typical experiment where increasing concentrations of HDL were incubated with CHO-hPON1 cells for 2, 4, or 7 h and then analyzed for activity with phenylacetate. Each point is the mean (± S.D.) of six determinations. B, double reciprocal plot of activity against HDL protein concentration for each incubation period.

**Fig. 6.** Analysis of cell-associated PON1. a, CHO-hPON1 cells; b, non-transfected CHO cells; c, CHO-apoAI cells transiently transfected with PON1; d, non-transfected CHO-apoAI cells. All were incubated with anti-PON1 antibody then FITC-labeled second antibody. Inset shows magnified area to demonstrate a punctate pattern of immunofluorescence. e, CHO-hPON1 cells after incubation with β-naphthylacetate and Fast Blue, which give an insoluble product due to hydrolysis by PON1. Note red staining of cells, and absence of staining from areas not containing cells. Inset shows accumulation of stain in pattern similar to immunofluorescence profile. f, non-transfected CHO cells treated as for e. Inset shows absence of precipitate on enlarged cells. g, transfected CHO-hPON1 cells after incubation with β-naphthylacetate and Fast Blue in the presence of EDTA. h, HuH-7 cells transiently transfected with PON1 and i, non-transfected HuH-7 cells treated as for a–d. White arrows indicate location of fluorescence/staining at surface of cells. Black arrows indicate permeabilized cells with extensive staining.
PON1 at the external face of the plasma membrane is a potential source of enzyme released into the medium. To test this possibility, CHO-PON1 cells were incubated in the presence and absence of hHDL and cell-bound activity measured. Cells had been previously treated with cycloheximide to block protein synthesis and prevent possible replacement of desorbed PON1 from intracellular sources. There was a significant decrease in cell-bound PON1 activity in the presence of HDL (Table II; \( p < 0.002, n = 15 \)). It correlated with the appearance of PON1 in the culture medium, as demonstrated by activity measurements and immunoblotting (not shown).

**PON1 Is Present in the Plasma Membrane of Transfected Hepatocytes**—To analyze the relevance to hepatocytes of the observations described above, the hepatocyte cell line HuH-7 was transiently transfectected with PON1. The presence of PON1 in the external membrane of the cells was first analyzed. Fig. 6h shows immunofluorescence of transfected cells probed with the anti-PON1 antibody. In non-permeabilized cells, fluorescence was located at the cell membrane in a punctate pattern comparable with that of stable-transfected CHO-hPON1 cells (Fig. 6a). Not all cells were stained, reflecting the partial and transient nature of the transfection. A second analysis examined cell-associated enzyme activity using the substrate, phenylacetate. With non-permeabilized HuH-7 cells, a significantly greater level of activity was observed to be associated with the PON1 transfected hepatocytes compared with non-transfected cells (Table II, \( p < 0.0001 \)). Localization of PON1 to the cell membrane was observed despite the ability of the HuH-7 cells to secrete apoA-I as shown by Western blotting of conditioned medium (Fig. 7, lane 5). Finally, in HuH-7 and CHO-apoAI cells transiently transfected with PON1, HDL was still capable of stimulating release of PON1 in the presence of co-secreted apoA-I (Fig. 7b), to an extent similar to that observed for CHO-PON1 cells (Fig. 1a).

**DISCUSSION**

The present study has focused on parameters that may influence the release of PON1 from cells, and the mechanism that is involved. It employed a model derived from stable transfection of CHO cells. The rationale was to avoid co-secretion of apoA-I and other apolipoproteins, as well as lipids, which could lead to formation of HDL-like complexes and thus confound interpretation of the observations. Our results suggest that in the absence of an appropriate acceptor, little PON1 is released from the cell. With an appropriate acceptor, up to 5-fold increases in PON1 release can be observed. The data clearly designate HDL as the predominant, physiological acceptor, which cannot be efficiently substituted by lipid poor apoA-I or A-II. Phospholipid complexes can also promote PON1 release, although LDL, which also have an important phospholipid content, were deficient in this respect. However, the presence of both apolipoprotein and phospholipid are necessary to promote release and maintain enzyme activity presumably by stabilizing the enzyme after its release. The study thus underlines the global importance of HDL to PON1 metabolism and function and emphasizes that their role may not be limited to providing a serum transport vehicle for the enzyme.

Our results establish that HDL increases PON1 activity both by promoting greater release of the peptide from cells and by stabilizing the enzyme. In this respect, phospholipids and apoA-I could have synergistic roles for PON1 function. Both appear necessary for optimal release and activity/stability of the enzyme. While apoA-I alone cannot promote PON1 release, we did note an apparent increase in PON1 specific activity immediately after secretion into medium containing lipid-poor apoA-I, suggesting a transient, positive influence on activity/stability. The observations that POPC vesicles and rHDLAII can promote release are interesting. They may offer one explanation for the presence of PON1 in apoA-I knockout models (30) and in human HDL deficiency syndromes (20). This is of clinical relevance as it suggests that HDL deficiency does not necessarily mean absence of an anti-atherogenic mechanism thought to depend on HDL. Our demonstration that PON1 can associate with protein-free phospholipid vesicles concurs with data from Sorenson et al. (30). Their elegant study, which underlined the importance of the hydrophobic tail of PON1 for its insertion into the HDL complex, also reported loss of activity from PON1-phospholipid vesicles, to a degree remarkably similar to our observations (23 versus 25% (our study) after an overnight incubation). We also observed a minor increase in specific activity when HDL was added to presecreted PON1. Our data are strikingly similar to and confirm those of Oda et al. (31). However, whether this observation is of physiological relevance is questionable in the light of our results, which suggest that PON1 may only be released in the presence of an appropriate acceptor. Nevertheless, the data are consistent with the possibility that increased specific activity may be an additional mechanism by which HDL could influence PON1 function.

We found that reconstituted HDL containing apoA-II were also able to stimulate PON1 release and stabilize activity. There is presently little data on the type of HDL particle with which PON1 is associated in vivo, although in early studies (19) we observed a preference for apoA-I containing HDL-particles. This does not exclude association of PON1 with A-II particles. In their in vitro studies, Sorenson et al. (30) reported loss of activity from apoA-II phospholipid vesicles. These observations may reflect basic differences in experimental design, which could implicate the secretory process. The present study exam-
ined transfer of PON1 from the producing cell to the acceptor complex. The preceding study (30) examined transfer of human serum-purified or presecreted, recombinant PON1 to acceptor complexes. It is possible that the secretory process favors correct insertion of PON1 into the acceptor complex. The latter may not be achieved by transfer of the enzyme between complexes, which could compromise its activity/stability. Another observation is of interest in this respect as we found that incubation with phospholipid vesicles or rHDL led to preferential association of PON1 with a distinct subpopulation. The enzyme did not simply associate with the bulk of rHDL, or POPC vesicles, as concluded previously (30). PON1 preferentially associated with larger sized vesicles. Thus, the secretory process may be of fundamental importance for defining the lipoprotein destination of secreted PON1 and thus optimizing activity/stability. It appears to favor larger sized complexes, which may have implications for PON1 secretion in clinical syndromes (e.g. diabetes) where HDL size tends to be diminished. The observation that the addition of free cholesterol to rHDL influences its capacity to promote PON1 release may also have clinical implications. Unesterified cholesterol partitions into the outer lipid layer in native lipoprotein complexes, and it can adversely affect lipoprotein function if present in elevated concentrations. Pathological changes to HDL function due to unesterified cholesterol were reported for diabetic patients (32). Our group and others (33, 34) have observed significantly lower concentrations of PON1 in diabetic patients.

Nothing is known about the mechanism of PON1 release in vitro. Our study highlights several interesting features, at least for this particular model. First, the peptide is inserted into the plasma membrane and is oriented toward the extracellular milieu. It does not have a sequence corresponding to a transmembrane domain and is probably present only at the external surface, presumably anchored by the hydrophobic tail. Second, loss of PON1 from the cell may involve, at least in part, desorption/release from the plasma membrane. Third, HDL (and the phospholipid vesicles) that stimulate enzyme release do so in a saturable, high affinity manner. The third point in particular would appear to militate against a process involving shedding of the enzyme and its absorption by available acceptor. The highly hydrophobic nature of the N-terminal sequence would also argue against such a mechanism. The latter would appear to necessitate a process that minimizes exposure of the tail to a hydrophilic environment. The kinetics of the appearance of PON1 in culture medium are compatible with a receptor-mediated process. In this context, the scavenger receptor SR-B1 may be of relevance. This HDL receptor functions as a docking membrane protein, allowing transient association of HDL with the cell surface (35). Several of the features of our model system for PON1 release mirror characteristics of the SR-B1 receptor. The receptor has a loose ligand specificity and can bind apoA-I and apoA-II containing HDL, as well as phospholipid vesicles (35, 36). Lipid-poor apoA-I does not appear to be a ligand for SR-B1. Finally, the estimated affinity constant for HDL-promoted PON1 release suggests high affinity binding to the CHO-hPON1 cells. Incidentally, the SR-B1 receptor was detected immunologically in the CHO-hPON1 line. The particular feature of SR-B1 function is to allow bi-directional transfer of hydrophobic molecules between lipoprotein and cell, without internalization of the lipoprotein particle. We hypothesize that PON1 may exploit this mechanism to allow secretion and correct insertion into the HDL complex.

Our observation concerning insertion of PON1 into the plasma membrane of transfected hepatocyte and CHO cells has interesting implications. The fact that it is oriented toward the external milieu and is active offers a potential mechanism for protection of cells from oxidized LDL or other oxidized lipids. It provides circumstantial evidence for an extended protective role of PON1. Further studies are warranted to determine whether the enzyme can also be transferred from HDL to cell membranes. Indeed, displacement of PON1 from HDL has already been described in vitro and in vivo under conditions of acute infection (16, 37). Acute phase proteins can remodel HDL composition, and while the phenomenon has been shown to decrease the anti-oxidant/anti-inflammatory capacity of the lipoprotein (16, 37), the question arises whether it could paradoxically benefit cell membranes by increasing their anti-oxidant capacity.

While the present study focused on a CHO model, PON1 is produced by the liver and thus an obvious question is the relevance of the observations to hepatic PON1. In a preliminary approach to the question, we wished to determine whether PON1 was inserted into the external plasma membrane of hepatocytes, thus presenting the structural features compatible with the release process that we have hypothesized. We were unable to identify a transformed hepatocyte line with suitable endogenous production of PON1 for such studies and thus employed transiently transfected hepatocytes. The results closely paralleled those observed with the CHO-hPON1 cells: the peptide was located in a punctate pattern at the external face of the HuH-7 cell membrane and, as with CHO-hPON1, it was enzymatically active. The data also underscore the fact that PON1 accumulates in the cell membrane despite the capacity of HuH-7 cells to secrete apoA-I (our results and Ref. 38) and confirm our observations with apoA-I-transfected CHO cells. Moreover, HDL stimulates PON1 release even when there is co-secretion of apoA-I.

Based on our observations, we hypothesize that release of the anti-oxidant enzyme, PON1, involves a docking process whereby acceptor complexes transiently associate with the cell membrane. HDL is the most appropriate, physiological acceptor, which can optimize enzyme activity/stability. We propose that a potential candidate for the docking molecule is the SR-B1 receptor. Several features of the PON1 secretory process mirror properties of the receptor. We speculate that the secre-
tory process is of fundamental importance for assuring the correct lipoprotein destination of PON1 and thus its functional efficiency. The results also indicate that modifications to HDL, as occur in several clinical conditions, may impair the capacity of the lipoprotein to facilitate PON1 release.

Acknowledgments—We gratefully thank the technical expertise of Barbara Kalix and the advice on immunofluorescence studies from Professor P. Meda and Dr. Veronique Serre-Beinier.

REFERENCES

1. Mackness, M. I., Arrol, S., Abbot, C., and Durrington, P. N. (1993) *Atherosclerosis* **104**, 129–135
2. Watson, A. D., Berliner, J. A., Hama, S. Y., La Du, B. N., Faull, K. F., Fogelman, A. M., and Navab, M. (1995) *J. Clin. Invest.* **96**, 2882–2891
3. Navab, M., Berliner, J. A., Subhanagounder, G., Hama, S., Lusis, A. J., Castellani, L. W., Reddy, S., Shi, J., Shi, W., Watson, A. D., Van Lenten, B. J., Vora, D., and Fogelman, A. M. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21**, 481–488
4. Shih, D. M., Xia, Y.-R., Wang, X. P., Miller, E., Castellani, L. W., Furlong, C., Costa, L. G., Fogelman, A. M., and Lusis, A. J. (1998) *J. Biol. Chem.* **273**, 265–271
5. Shih, D. M., Xia, Y.-R., Wang, X. P., Miller, E., Castellani, L. W., Furlong, C., Costa, L. G., Fogelman, A. M., and Lusis, A. J. (1998) *Nature* **394**, 284–287
6. Shih, D. M., Xia, Y.-R., Wang, X. P., Miller, E., Castellani, L. W., Subhanagounder, G., Cherestre, H., Faull, K. F., Berliner, J. A., Witztum, J. L., and Lusis, A. J. (2000) *J. Biol. Chem.* **275**, 17527–17535
7. Ruiz, J., Blancé, H., James, R. W., Blatter, M. C., Chapentier, G., Morabia, A., Passa, P., and Foguel, P. (1995) *Lancet* **346**, 869–872
8. Blatter Garin, M.-C., James, R. W., Dussouix, P., Blancé, H., Passa, P., Foguel, P., and Ruiz, J. (1997) *J. Clin. Invest.* **99**, 62–66
9. Serrato, M., and Marian, A. J. (1995) *J. Clin. Invest.* **95**, 3005–3008
10. Oda, M. N., Bielicki, J. K., Berger, T., and Forte, T. M. (2001) *Biochemistry* **40**, 1710–1718
11. Pfohl, M., Koch, M., Enderle, M. D., Kuhn, R., Fullhase, J., Karsch, K. R., and Haring, H. U. (1999) *Diabetes* **48**, 623–627
12. Antikkainen, M., Murtoniemi, S., Syvanne, M., Pahlman, R., Talvanainen, E., Jauhainen, M., Frick, M. H., and Ehaholm, C. (1996) *J. Clin. Invest.* **98**, 883–885
13. Gardemann, A., Philipp, M., Hess, K., Katz, N., Tillmanns, H., and Haberbosch, W. (2000) *Atherosclerosis* **152**, 421–431
14. Leviev, I., and James, R. W. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20**, 516–521
15. James, R. W., Leviev, I., Ruiz, J., Passa, P., Foguel, P., and Garin, M.-C. (2000) *Diabetes* **49**, 1390–1393
16. Van Lenten, B. J., Hama, S. Y., de Beer, F. C., Stafforini, D. M., McIntyre, T. M., Prescott, S. M., La Du, B. N., Fogelman, A. M., and Navab, M. (1995) *J. Clin. Invest.* **96**, 2758–2767
17. Aviram, M., Rosenblat, M., Biagier, C. L., Newton, R. S., Prime-Parma, S. L., and La Du, B. N. (1998) *J. Clin. Invest.* **101**, 1551–1560
18. Shih, D. M., Gu, L., Hama, S., Xi, Y.-R., Navab, M., Fogelman, A. M., and Lusis, A. J. (1996) *J. Clin. Invest.* **97**, 1630–1639
19. Blatter Garin, M.-C., James, R. W., Messmer, S., Barja, F., and Pometta, D. (1993) *Eur. J. Biochem.* **211**, 871–879
20. James, R. W., Blatter Garin, M. C., Calabresi, L., Miccoli, R., von Eckardstein, A., Tilly-Kiesi, M., Taskinen, M. R., Assmann, G., and Franceschini, G. (1998) *Atherosclerosis* **139**, 77–82
21. Leviev, I., Deakin, S., and James, R. W. (2001) *J. Lipid Res.* **42**, 528–535
22. James, R. W., Hochstrasser, D., Tissot, J.-D., Funk, M., Appel, R., Barja, F., Pellegrini, C., Muller, A. F., and Pometta, D. (1988) *J. Lipid Res.* **29**, 1557–1571
23. Havel, R. J., Eder, H. A., and Bragton, J. H. (1955) *J. Clin. Invest.* **34**, 1345–1353
24. Calabresi, L., Vecchio, G., Frigerio, F., Vavassori, L., Sirtori, C. R., and Franceschini, G. (1997) *Biochemistry* **36**, 12428–12433
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
26. Hanks, J. H., and Wallace, R. E. (1949) *Proc. Soc. Expil. Biol. Med.* **71**, 196–200
27. Pimplikar, S. W., and Huttner, W. B. (1992) *J. Biol. Chem.* **267**, 4110–4118
28. Blatter Garin, M.-C., Abbot, C., Messmer, S., Mackness, M. I., Durrington, P. N., Pometta, D., and James, R. W. (1994) *Biochem. J.* **304**, 549–554
29. Miller, R. B., and Karn, R. C. (1980) *J. Lipid Res.* **21**, 812–818
30. Abbott, C. A., Mackness, M. I., Kumar, S., Boulton, A. J., and Durrington, P. N. (1995) *Atherosclerosis* **111**, 247–250
31. Oda, M. N., Bielicki, J. K., Berger, T., and Forte, T. M. (2001) *Biochemistry* **40**, 1710–1718
32. Bagdade, J. D., and Subbaiah, P. V. (1989) *J. Lab. Clin. Med.* **113**, 235–240
33. Abbott, C. A., Mackness, M. I., Kumar, S., Boulton, A. J., and Durrington, P. N. (1995) *Atherosclerosis* **111**, 247–250
34. Boemi, M., Leviev, I., Siriola, C., Pieri, C., Marra, M., and James, R. W. (2001) *Atherosclerosis* **155**, 229–235
35. Krieger, M. (1999) *Annu. Rev. Biochem.* **68**, 523–558
36. Trigatti, B. L., Rigotti, A., and Braun, A. (2000) *Biochim. Biophys. Acta* **1529**, 276–286
37. Van Lenten, B. J., Wagner, A. C., Nayak, D. P., Hama, S., Navab, M., and Fogelman, A. M. (2001) *Circulation* **103**, 2282–2288
38. Yamamoto, T., Suda, M., Moriwaki, Y., Takahashi, S., and Higashino, K. (1990) *Biochim. Biophys. Acta* **1047**, 49–56
Enzymatically Active Paraoxonase-1 Is Located at the External Membrane of Producing Cells and Released by a High Affinity, Saturable, Desorption Mechanism
Sara Deakin, Ilia Leviev, Monica Gomaraschi, Laura Calabresi, Guido Franceschini and Richard W. James

J. Biol. Chem. 2002, 277:4301-4308.
doi: 10.1074/jbc.M107440200 originally published online November 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107440200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 38 references, 14 of which can be accessed free at
http://www.jbc.org/content/277/6/4301.full.html#ref-list-1