Cysteine Substitutions in Apolipoprotein A-I Primary Structure Modulate Paraoxonase Activity†

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ABSTRACT: Paraoxonase (PON) is transported primarily on apolipoprotein A-I (apoA-I) -containing high-density lipoprotein (HDL) and is thought to protect against early atherogenic events including low-density lipoprotein (LDL) oxidation and monocyte migration. It has been proposed that apoA-I may be necessary for PON's association with plasma HDL. On the basis of this, we examined the effect of apoA-I on PON's enzymatic activity and its ability to associate with HDL. Additionally, we examined whether changes in apoA-I primary structure (cysteine substitution mutations) could modulate these effects. Chinese hamster ovary cells stably transfected with human PON1A cDNA were incubated in the presence and absence of recombinant wild-type apoA-I (apoA-I_{WT}) and specific Cys substitution mutations. Extracellular accumulation of PON activity in the presence of apoA-I_{WT} was 0.095 ± 0.013 unit/mg of cell protein (n = 7) compared to 0.034 ± 0.010 unit/mg of cell protein in the absence of apoA-I (n = 7), a 2.79-fold increase in activity when apoA-I was incubated with the cells. Lipid-free apoA-I did not increase PON activity, while preformed nascent HDL increased PON activity only 30%, suggesting that maximal PON activity is lipid-dependent and requires coassembly of PON and apoA-I on nascent HDL. The cysteine mutations R10C, R27C, and R61C significantly increased (p < 0.01) PON activity 32.6% ± 14.7%, 31.6% ± 18.9%, and 27.4% ± 20%, respectively, over that of wild type (WT). No changes in PON activity were observed with apoA-I cysteine substitution mutations in the C-terminal portion of the protein. The data suggest that, for optimal PON activity, coassembly of the enzyme onto nascent HDL is required and that the N-terminal region of apoA-I may be important in the assembly process.

Elevated HDL[1] concentration has been correlated with a reduced risk for coronary artery disease (CAD). HDL particles are thought to possess the ability to attenuate the initiation and progression of atherosclerotic lesions (*1*−*3*). It has been proposed that the protective effects of HDL are related, in part, to its ability to inhibit the formation of minimally oxidized LDL, thus preventing the accumulation of proinflammatory mediators associated with the onset of atherogenesis. This antioxidative activity is ascribed to the enzyme PON, which is exclusively transported on plasma HDL (*4*−*7*).

PON is a 43 kDa glycoprotein with a broad specificity class A esterase activity (*8*). Recent studies suggest that this enzyme can hydrolyze bioactive oxidized phospholipids (*6*). In vitro studies by Mackness et al. (*5*) have demonstrated that HDL with PON is capable of attenuating the production of lipid hydroperoxides on LDL and the formation of minimally oxidized LDL. In PON knockout mice there is an accumulation of lipid hydroperoxides on LDL, and these mice are more susceptible to diet-induced atherogenesis (*9*). Furthermore, in humans, low concentrations of PON in plasma have been correlated with an increased risk for CAD (*10, 11*). Such studies suggest an important role for HDL−PON in the protection of LDL from oxidation and the concomitant protection of the artery wall from atherogenesis. It is likely that increased transport and/or stability of the PON enzyme on HDL would increase HDL's protective properties.

ApoA-I is the major structural protein on HDL. It consists of a series of amphipathic helices that are functionally important for protein−lipid interactions as well as protein−protein interactions. The carboxy terminus of apoA-I has high lipid-binding capacity, while the amino terminus has limited lipid-binding capacity but may be important in protein−protein interaction (*12, 13*). ApoA-I is largely responsible for mediating HDL assembly and is a determinant of HDL structure and composition. Previous studies showed that, during isolation, PON copurifies with apoA-I (*14*), while immunoaffinity chromatography studies indicate that PON can be found on a specific subpopulation of HDL containing apoA-I without apoA-II (*15*). Both these observations suggest that there may be direct interactions between PON and apoA-I. Recent studies by Sorenson et al. (*16*), using recombinant WT PON and a PON mutant lacking the hydrophobic

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[1] Abbreviations: apoA-I, apolipoprotein A-I; PON, paraoxonase; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CHO, Chinese hamster ovary cell; EYPC, egg yolk phosphatidylcholine; TBS, Tris-buffered saline; WT, wild type.

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N-terminal domain, suggested that PON interacts with HDL primarily through interactions of this hydrophobic moiety with HDL phospholipid. ApoA-I did enhance enzyme stability, again suggesting that on the HDL particles there may be PON−apoA-I interactions. Early events in the assembly of PON onto HDL are not well understood; hence, the present study examines this process with Chinese hamster ovary (CHO) cells stably transfected with human PON1A cDNA (CHO−PON).

Despite the strong association between HDL and PON, deficiencies in HDL and apoA-I show variable PON activity and mass. Homozygous Tangiers patients and apoA-I_{Pisa} patients have apoA-I concentrations <5% of normal, and some of these individuals have documented coronary artery disease (*17, 18*). This condition is associated with a 60−75% decrease in PON mass and PON arylesterase activity (*19*); potentially the reduction in PON may be a contributing factor to the coronary heart disease noted in the patients. Patients heterozygous for the Lys107 → 0 mutation (apoA-$I_{Helsinki}$) (*20*) have a 30% reduction in apoA-I and approximately 40% reduction in PON plasma concentration (*19*), and some subjects have documented coronary disease. Carriers of the apoA-I_{Milano} trait characterized by the presence of a sulfhydryl group in apoA-I (Arg173 → Cys mutation) (*21*) have substantial reductions in apoA-I (approximately 50%) but have no predisposition for CAD. These patients have PON mass and activity similar to normal subjects (*19*). Another Arg → Cys variant, Arg151 → Cys (apoA-I_{Paris}) (*22*), has low HDL cholesterol and apoA-I without increased CAD. In this case PON plasma activity and concentration were not evaluated. Like apoA-I_{Milano} and apoA-I_{Paris}, PON has a free sulfhydryl group, and site-directed mutagenesis has ruled out the possibility that this sulfhydryl group is part of the enzyme's esterase catalytic site (*23*). Potentially the presence of a Cys in apoA-I_{Milano} (and by extension apoA-I_{Paris}) could alter HDL, leading to increased PON stability or activity. This could explain, in part, the absence of premature CAD in these carriers.

Previous studies from our laboratory (*24*) have demonstrated that lipid-free apoA-I recruits phospholipid and cholesterol from CHO cells in an energy-dependent fashion. Exposure of exogenous apoA-I thus promotes the specific release of lipids to the protein and results in the formation of discoidal apoA-I−lipid complexes referred to as nascent HDL. This process has recently been shown to involve the ATP-binding cassette protein A-1 (*25*−*27*). In the present study we used stably transfected CHO cells expressing human serum PON incubated with lipid-free apoA-I to determine whether nascent HDL assembly was linked to the initial transport and optimal activity of the enzyme. As it is known that the apoA-I_{Milano} mutation affects its lipid binding properties (*28, 29*), we examined the capacity of this variant, as well as other artificial Cys substitution mutants, to mediate PON secretion and activity. To determine whether the position of the Cys mutation can modulate PON activity, substitutions were made in both the N-and C-termini. Results suggest that HDL assembly is not required for accumulation of PON protein in the culture medium but is required for optimal PON arylesterase activity. Moreover, results of the apoA-I mutagenesis studies revealed that some Cys substitutions in the N-terminal region, but not the C-terminal region, modulate PON-specific activity. Together these data suggest that apoA-I may be in direct contact with PON on the HDL particle and that this contact is HDL assembly-dependent.

MATERIALS AND METHODS

Transfection of CHO Cells with Human PON cDNA. The human PON1A cDNA was kindly provided by Dr. Bert La Du and was subcloned into the pcDNA3 vector (InVitrogen Inc., Carlsbad, CA). CHO-K1 cells (~400,000 cells/mL) were transfected by electroporation (10 μg of vector, 250 V, 20 ms discharge). Screening for PON1A expression was performed by diluting cells to 4−5 cells/mL and culturing in 96-well plates in McCoy's 5A medium plus 10% heat-inactivated fetal bovine serum (FBS) and G418 antibiotic (800 μg/mL) as a selective agent. The media from wells containing single colonies were individually harvested, slot-blotted onto nitrocellulose, and probed with anti-PON antiserum. Immunoreactive cells expressing arylesterase activity were further examined by SDS−PAGE Western blot for the expression of full-length PON1A protein (approximate molecular mass of 43 kDa). The stably transfected clone chosen for this work expressed arylesterase activity at 80 units/L.

Incubation of CHO−PON Cells. CHO−PON cells were grown in T-175 cell culture flasks containing McCoy's 5A medium plus 10% FBS. At confluency, the cells were first rinsed three times with Hanks' balanced salt solution and then incubated for 24 h in serum-free medium alone or in the presence of 0.5−20 μg/mL apoA-I (WT and mutants). The harvested conditioned medium was centrifuged briefly to eliminate cellular debris and concentrated approximately 100-fold by ultrafiltration (10,000 MW cutoff) and supplemented with 0.05 mg/mL gentamicin sulfate, 1 mM benzamidine, and 1 mM $CaCl_2$. Adherent cells in the flask were harvested and protein mass was determined by Markwell's modified Lowry method (*30*).

Production of Recombinant ApoA-I Protein. Human apoA-I cDNA was modified by primer-directed mutagenesis to encode a His-6 affinity tag N-terminal extension by use of the synthetic oligonucleotide 5′ ACC CAT ATG CAT CAC CAT CAC CAT CAC ATC GAA GGT CGT GAC GAG CCA CCG CAG 3′. The protein encoded by the resulting cDNA was N-terminally modified with the amino acid sequence Met-$(His)_6$-Ile-Glu-Gly-Arg, which encodes a His-6 affinity tag and factor X_a proteolytic cleavage site. The apoA-I cDNA sequence was confirmed by dideoxy automated fluorescent sequencing and subcloned into the pET-20b bacterial expression vector, acquired from Novagen, Inc. (Madison, WI), to produce the human apoA-I bacterial expression vector phAIex.

The plasmid was transformed into the *Escherichia coli* strain BL21 (DE-3) pLysS. *E. coli* harboring the phAIex plasmid were grown to 0.6 OD_{600} in NCZYM medium containing 100 μg/mL ampicillin and induced by the addition of IPTG (0.4 mM), and the cells were cultured for an additional 3 h at 37 °C. The cells were harvested by centrifugation at 10000*g* for 15 min, resuspended in B-PER bacterial protein extraction reagent (Pierce; Rockford, IL), and lysed by sonication. Cellular debris was removed by centrifugation at 10000*g* for 15 min. Cleared lysates were mixed with an equal volume of 2× column loading buffer (40 mM $NaPO_4$, 1 M NaCl, and 6 M guanidine hydrochlo-

ride, pH 7.4) and passed through a 5 mL His-Trap chelating column (Pharmacia Inc.) preloaded with 0.1 M $NiSO_4$. The column was washed with 25 mL of 1× loading buffer followed by 25 mL of wash buffer (20 mM $NaPO_4$ and 0.5 M NaCl, pH 7.4). ApoA-I was eluted from the column with 25 mL of elution buffer (20 mM $NaPO_4$, 0.5 M NaCl, and 0.5 M imidazole, pH 7.4) in 0.5 mL fractions. The elution profile was determined spectraphotometrically at A_{280} (corrected for imidazole absorbance) and the A_{280} peak was pooled. The pooled eluted protein was dialyzed extensively against Tris-buffered saline (TBS; 20 mM Tris and 150 mM NaCl, pH 8.0) supplemented with 1 mM benzamidine and 1 mM EDTA, filter-sterilized, and stored at 4 °C or frozen at −70 °C. Phospholipid analysis carried out on the purified protein showed that it was lipid-free.

The Cys substitution mutations within apoA-I cDNA (R10C, R27C, R61C, K96C, R123C, R131C, R151C, R173C, R215C, and R238C) were created either by primer-directed PCR mutagenesis or by the Mega-Primer PCR method (*31*). The mutations were verified by dideoxy automated fluorescent sequencing.

Bacterial Expression of Human PON1A for Antibody Production. The PON1A cDNA was subcloned into the pET-20b bacterial expression vector and expressed in *E. coli* as above. We found that bacterially expressed human PON was extremely insoluble and took advantage of this property by enriching human PON through a series of detergent washes. The bacterial extract was centrifuged at 10000*g* for 15 min and the pellet containing human PON1A protein was retained. The pellet was resuspended in TBS and 1% Triton X-100 and recentrifuged at 10000*g* for 15 min. The Triton X-100 extraction pellet was resuspended in TBS and 1% SDS and centrifuged at 10000*g* for 15 min. The supernatant containing human PON1A protein was retained. The enriched solubilized human PON1A protein was analyzed by SDS−PAGE and determined to be near homogeneity (>95%). The solubilized protein was subjected to preparative SDS−PAGE and the band containing purified human PON1A was electroeluted. The protein was quantified by the method of Markwell (*30*).

Approximately 100 μg of purified human PON1A was emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO) and injected into a female Alpine goat; at week 4 the goat was boosted with a second injection of purified human PON1A in incomplete Freund's adjuvant. Blood was drawn at weeks 4−9 postinoculation and examined for anti-PON, using as the test antigen human PON1A isolated from plasma kindly provided by Dr. Bert La Du. The serum was isolated from the blood, supplemented with sodium azide to 0.02%, filter-sterilized, and stored at −70 °C. The antiserum did not cross-react with human apoA-I, apoA-II, or albumin nor with mouse apoA-I or PON.

Analysis of PON Arylesterase Activity. PON activity was measured as arylesterase activity with phenylacetate as substrate in the presence of 1 mM $CaCl_2$, as described by Gan et al. (*14*). Activity was determined by monitoring the increase in absorption at 270 nm for 60 s. One unit of arylesterase activity is equal to 1 μmol of phenylacetate hydrolyzed/min.

Isolation and Analysis of Lipidated Complexes. Complexes resulting from incubation of CHO−PON cells with apoA-I were ultracentrifugally isolated at $d = 1.063-1.21$ g/mL (*32*),

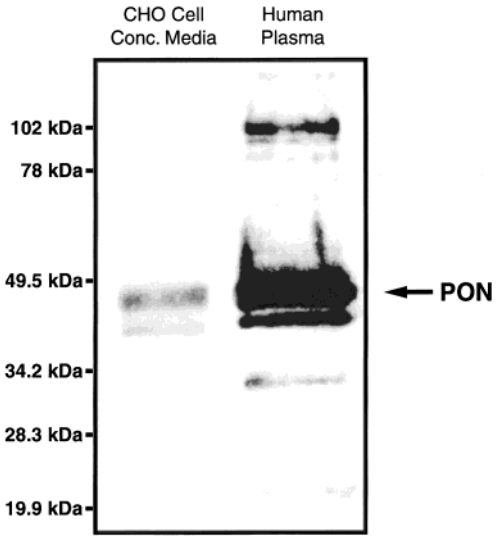

FIGURE 1: SDS−PAGE anti-PON Western Blot of CHO cell-secreted PON. Transfected CHO cell-conditioned medium was concentrated 200-fold. A 10 μL aliquot of the concentrated conditioned medium was loaded onto a 4−20% polyacrylamide nonreduced SDS gel, transferred to nitrocellulose, and probed with goat anti-serum to human PON. Human PON isolated from plasma (kindly provided by Dr. La Du) was run for comparison. The blot demonstrates that transfected CHO cells secrete full-length PON into the medium of the same molecular weight as the major band seen in human plasma PON.

by use of the TL100 ultracentrifuge (100.2 rotor) with 0.5 mL tubes. Concentrated 24 h conditioned medium (500 μL) was adjusted to $d = 1.063$ g/mL and centrifuged at 100,000 rpm for 3.5 h at 10 °C; the top 167 μL consisting of the $d < 1.063$ g/mL fraction was removed. The infranatant was brought to $d = 1.21$ g/mL with NaBr and centrifuged at 100,000 rpm for 5.25 h at 10 °C. The top 167 μL ($d = 1.063-1.21$ g/mL) fraction was removed and the remaining $d > 1.21$ g/mL fraction collected. The fractions were dialyzed overnight against TBS (pH 7.5) supplemented with 1 mM $CaCl_2$ at 4 °C. The arylesterase activity and total protein in each fraction was quantified.

SDS−PAGE and Western Blot Analyses. Proteins were visualized on precast 4−20% SDS−polyacrylamide gels (Novex, San Diego, CA) according to the procedure of Laemmli (*33*). For Western blot analysis, proteins were transferred to nitrocellulose. Blots were probed with antibodies specific for either human apoA-I (mouse monoclonal antibody, Chemicon Inc.) or human PON (goat anti-sera).

The presence of disulfide bond formation between Cys-substituted apoA-I and PON was determined by nonreducing SDS−PAGE analysis. Formation of Cys-substituted apoA-I−PON heterodimer was assessed by the presence or absence of a 70 kDa band immunoreactive with PON antibody.

Preparation of Reconstituted EYPC/ApoA-I Complexes. Discoidal complexes of egg yolk phosphatidylcholine (EYPC) and apoA-I were prepared by the cholate dialysis method as described by Nichols et al. (*34, 35*). Briefly, EYPC, apoA-I and sodium cholate were combined at mole ratios of 80:1:270 in 20 mM Tris-buffered (pH = 8.0) saline−EDTA and incubated (4° C) for 18−20 h. The mixture was then dialyzed extensively in this same buffer for several days to remove the sodium cholate. Nondenaturing gradient gel electrophoresis showed that this procedure resulted in the formation of a homogeneous preparation of 9.5 nm particles.

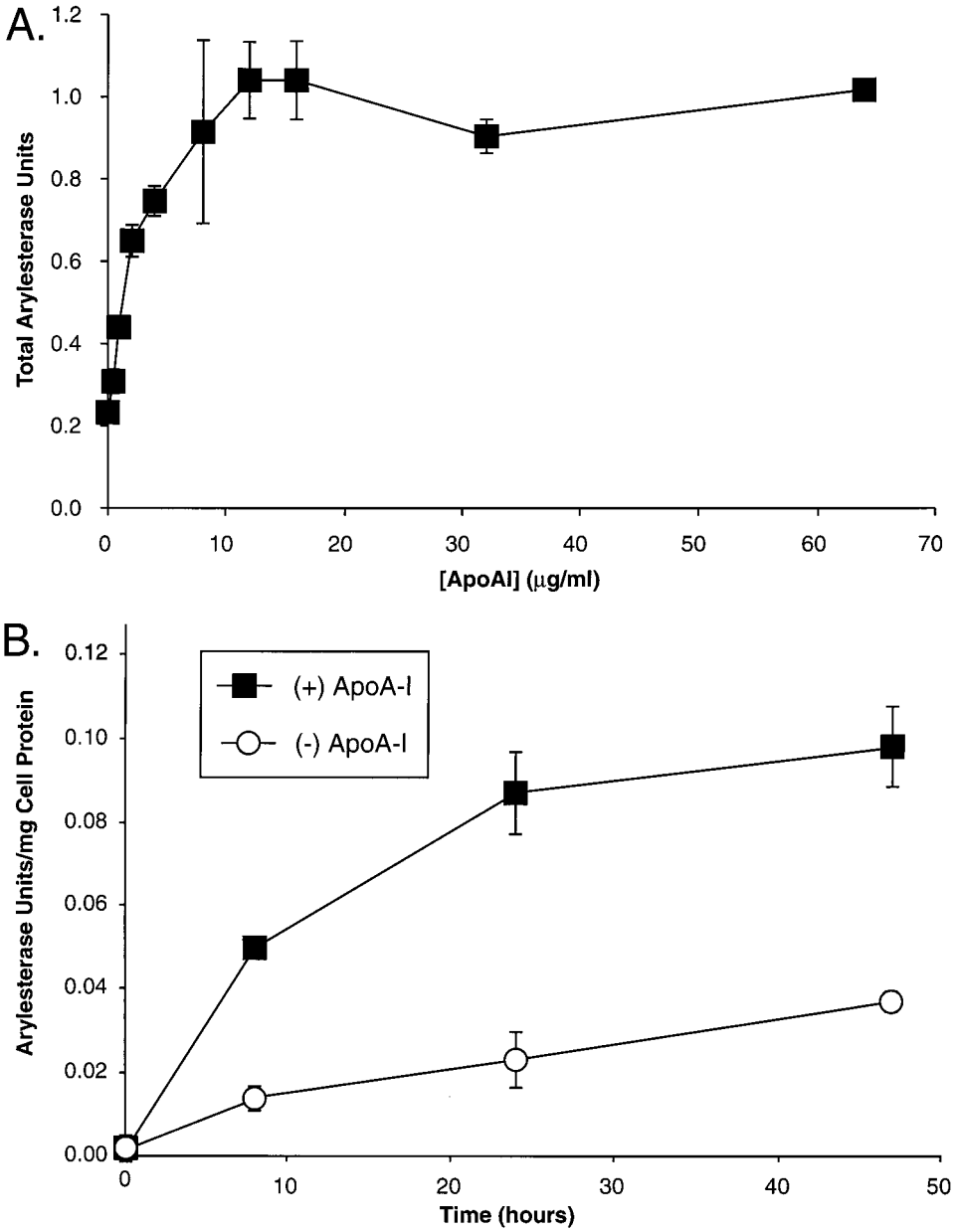

FIGURE 2: Effect of apoA-I on PON production and activity. The dependence of PON activity on apoA-I concentration is shown in panel A. The secreted PON activity is maximal at approximately 12 μg/mL apoA-I ($n = 4$). The time course of PON production in the absence and presence of 10 μg/mL apoA-I is shown in panel B. Conditioned medium was collected at 0, 8, 24, and 48 h and concentrated 200-fold. PON activity was normalized to cell protein. The level of secreted arylesterase activity was significantly increased in the presence of apoA-I ($n = 5$).

RESULTS

Expression of PON Activity in the Presence and Absence of ApoA-I. Human PON isolated from plasma is a glycoprotein of approximately 43 kDa; thus we examined PON secreted into CHO−PON cell medium to determine whether these cells produced a protein similar to that of human plasma. As seen in Figure 1, the secreted PON is similar in size to the plasma enzyme, suggesting that PON from CHO cells is fully glycosylated.

It has been suggested that PON may interact with apoA-I; thus the effect of lipid-free apoA-I added to CHO−PON cell medium during a 24 h incubation was examined. ApoA-I increases PON activity in the medium in a concentration-dependent manner as shown in Figure 2A. The maximal response was at a concentration of approximately 12 μg/mL. This concentration dependence is similar to that observed for apoA-I lipidation previously reported by Forte et al. (*24*), where CHO cells incubated in the presence of apoA-I formed nascent particles with a maximum percent apoA-I lipidation at 10 μg/mL. To confirm that the elevated PON activity was not specific to our recombinant apoA-I, apoA-I_{WT} isolated from human plasma was also examined and gave similar results (data not shown). To determine whether PON secretion was linear with time, CHO−PON cells were incubated in the absence and presence of 10 μg/mL apoA-I for up to 48 h. As seen in Figure 2B, in the absence of apoA-I, PON activity in the medium was linear over this time period; at 24 h arylesterase was 0.024 ± 0.009 unit/mg of cell protein ($n = 3$). In the presence of apoA-I, there is a significant increase in PON activity in the medium;

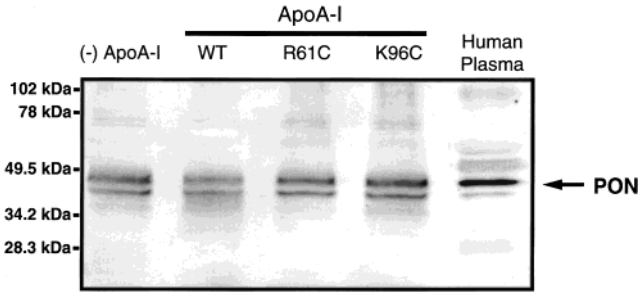

FIGURE 3: SDS−PAGE anti-PON Western blot indicates that the presence of apoA-I in the medium does not increase PON secretion. The relative mass of PON produced in the presence and absence of apoA-I_{WT}, as well as the Cys mutants R61C and K96C, was examined on a 4−20% polyacrylamide nonreduced SDS gel with 10 μL of concentrated 24 h conditioned medium. Proteins were transferred to nitrocellulose membranes and probed with antisera to human PON. The positions of the molecular weight standards are shown on the left. Purified plasma PON was used as a standard to confirm the position of PON. The amount of PON expressed in the medium was similar whether apoA-I was present or absent. Unique molecular weight variants of PON near 70 kDa did not arise in the presence of apoA-I Cys mutants, suggesting that apoA-I_{cys}–PON covalently linked heterodimers do not form.

arylesterase activity at 24 h was 0.087 ± 0.013 unit/mg of cell protein (n = 3).

To ascertain whether increased PON activity reflected increased PON secretion or increased specific activity of the enzyme, an equivalent amount of total protein from concentrated conditioned medium from cells incubated in the absence of apoA-I as well as in the presence of apoA-I_{WT} and two cysteine mutations, R61C and K96C, was loaded onto a 4−20% polyacrylamide gel, electrophoresed, transferred, and immunoprobed with anti-PON antibody. Protein mass, as indicated by immunoreactivity to PON (Figure 3), is approximately the same for PON accumulating in the absence of apoA-I as in the presence of apoA-I, suggesting that apoA-I increases specific activity of the enzyme and not its mass.

ApoA-I-Induced Elevation in PON Activity Is Lipid-Dependent. The results in Figure 2 suggest that PON activity was dependent on the assembly of nascent HDL. However, we could not rule out that lipid-free apoA-I may interact with secreted PON and independently increase activity. To differentiate between these possibilities, 24 h concentrated conditioned medium was exposed, postculture, to lipid-free apoA-I. As shown in Figure 4, the postincubation addition of lipid-free apoA-I to conditioned medium possessing PON activity (i.e., absence of cells) did not augment enzyme activity, whereas culturing of CHO−PON cells with apoA-I produced a 2.1-fold increase in arylesterase activity.

To verify that apoA-I nascent particles were indeed formed in the CHO−PON cell culture medium during 24 h incubation of the cells with 10 μg/mL apoA-I, conditioned medium was subjected to ultracentrifugation and the d = 1.063−1.21 g/mL fraction was isolated and examined by electron microscopy. The particles floating at this density consisted of discoidal particles 10−20 nm in diameter (data not shown).

To ascertain whether all PON activity is associated with lipidated complexes during incubation of CHO−PON cells with apoA-I, the distribution of PON activity in ultracentrifugal fractions was measured. No PON activity was detected in the d < 1.063 g/mL fraction. As seen in Figure 5, approximately 43% of the total arylesterase activity was associated with the nascent HDL top fraction floating at d = 1.063−1.21 g/mL; the remainder resided in the d > 1.21 g/mL protein-rich bottom fraction. Although we cannot completely rule out the displacement of some PON activity from lipidated complexes during ultracentrifugation, the specific activity for arylesterase is approximately 15-fold greater in the lipid-rich HDL fraction than in the d > 1.21 g/mL lipid-poor bottom fraction. These data suggest that although half of the enzyme activity is associated with the lipid-poor fraction, the nascent HDL fraction is a more effective environment for PON activity. These results support the premise that PON is associated with nascent HDL and that arylesterase activity is significantly higher (2-fold) in the presence of lipidated apoA-I complexes.

CHO−PON cell incubation studies suggest that PON is incorporated into nascent HDL particles during their assembly. To determine whether PON activity is equally activated when incubated with preformed apoA-I/phospholipid complexes, CHO−PON conditioned medium obtained in the absence of apoA-I was incubated with apoA-I/EYPC complexes. PON activity was 1.44 ± 0.018 units/mL (n = 3) for medium incubated with apoA-I/EYPC complexes, compared to 1.05 ± 0.010 units/mL for cells incubated in the absence of apoA-I. This represents a modest 37% ± 1.7% increase in activity compared to a 203% ± 16.3% increase when cells were grown in the presence of apoA-I (2.13 ± 0.35 units/mL; n = 3). These data support the premise that simultaneous lipidation of PON enzyme and apoA-I is required for optimal arylesterase activity, whereas exposure of PON to preformed lipid complexes only partially activates the enzyme. Since we could not rule out the possibility that the physical−chemical properties of apoA-I/EYPC complexes were not appropriate to support optimal enzyme activity, nascent HDL were isolated from nontransfected CHO cells incubated in the presence of apoA-I according to the method of Forte et al. (*24*). Under these conditions, there was only a 30% increase in PON activity (data not shown), ruling out that the diminished PON activity response was specific to apoA-I/EYPC complexes.

Effect of ApoA-I Cysteine Substitution Mutations on PON Arylesterase Activity. Because PON possesses a free sulfhydryl group, we examined the possibility that Cys substitutions in apoA-I, particularly at residues 151 (apoA-I_{Paris}) and 173 (apoA-I_{Milano}), may result in increased PON arylesterase activity, potentially through stabilization of the PON−HDL complex. Additionally, a series of mutations were made throughout the apoA-I molecule, including the following: R10C, R27C, R61C, K96C, R123C, R131C, R151C, R173C, R215C, and R238C. Recombinant apoA-I_{WT} and Cys substituted apoA-I variants were examined by reduced and nonreduced SDS−PAGE to assess the degree of dimerization. As noted in Figure 6A, under nonreducing conditions, the degree of dimerization was variable depending on the site of Cys substitution. Upon reduction, all bands migrate as a single band (Figure 6B), indicating that the additional bands arose from conformational variants that exist in the covalently linked dimer. Additionally, the dimer form of apoA-I Cys-containing mutants migrated with increasing apparent molecular weight up to residue 131 and with

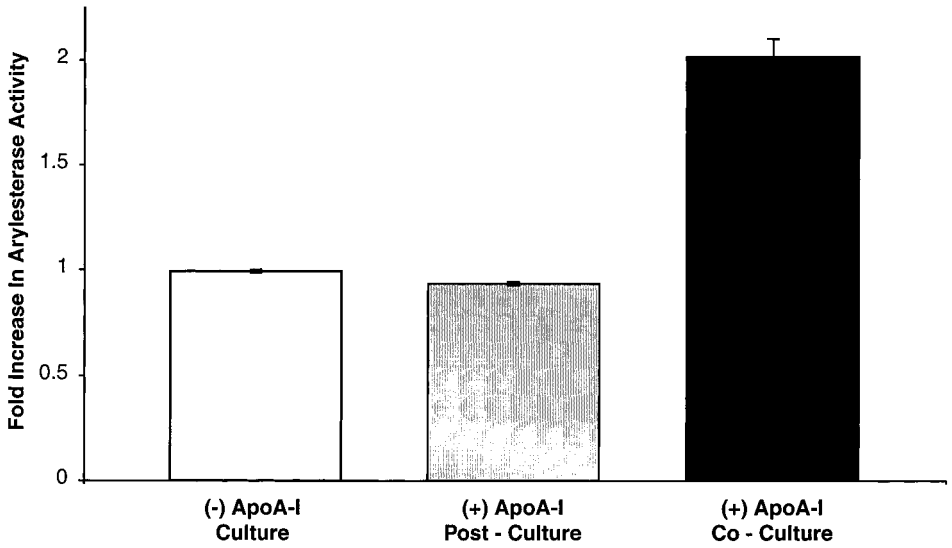

FIGURE 4: Optimal PON activity requires apoA-I lipidation. CHO cells were cultured overnight in serum-free medium both in the absence (white bar = baseline) and presence (black bar) of 10 μg/mL apoA-I. The latter shows a 203% ± 16% increase in activity over cells grown in the absence of apoA-I. To rule out that lipid-free apoA-I is sufficient to increase arylesterase activity, the baseline medium was incubated for 3 h at 37 °C, with 10 μg/mL apoA-I postculture but no increase in enzyme activity was noted (gray bar, 94.4% ± 1.5%). Values represent the means of three experiments.

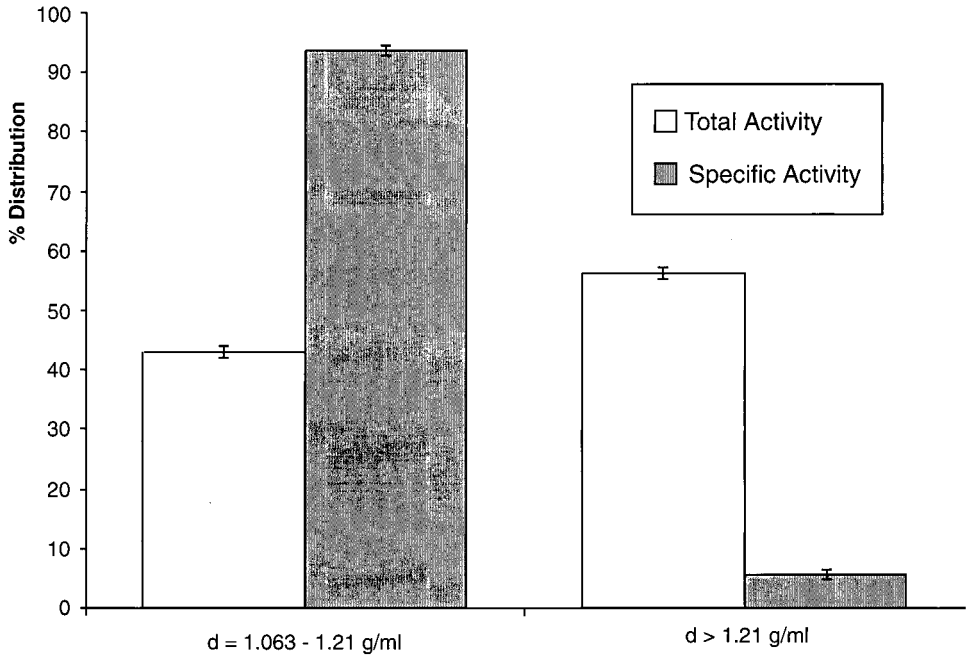

FIGURE 5: Distribution of PON activity in nascent HDL particles versus the lipid-free fraction. The distribution of arylesterase activity was assessed after ultracentrifugal fractionation of concentrated conditioned medium. The conditioned medium from CHO−PON cells was concentrated 200-fold and subjected to ultracentrifugal fractionation as described under Materials and Methods. The $d = 1.063-1.21$ g/mL and $d > 1.21$ g/mL fractions were collected, representing the HDL and lipid-poor fractions, respectively. Total activity (white bar) and specific activity (units per milligram of protein, gray bar) were determined. Data represents the mean ± standard deviation of three experiments. A larger fraction of the PON arylesterase activity was associated with the lipid-poor $d > 1.21$ g/mL fraction compared to the $d = 1.063-1.21$ g/mL fractions (56.6 ± 0.97 versus 43.4 ± 0.97 total units, respectively). Arylesterase specific activity is nearly 15-fold higher in the HDL $d = 1.063-1.21$ g/mL fraction compared to the $d > 1.21$ g/mL fraction (94% ± 0.76% versus 6.0% ± 0.76%).

decreasing molecular weight thereafter, while not affecting the apparent molecular weight of the monomeric state. This observation suggests that the covalently linked dimer is in a more elongated conformation when the Cys is positioned centrally within the apoA-I primary sequence, consistent with the elongated two-helix bundle model for apoA-I lipid-free structure proposed by Rogers et al. (*36, 37*).

ApoA-I Cys variants were incubated with CHO−PON cells to investigate their ability to stimulate PON activity. Substitutions in the N-terminal third of apoA-I significantly elevated arylesterase activity approximately 30% in R10C, R27C, and R61C mutants (0.126 ± 0.014, 0.125 ± 0.018, and 0.121 ± 0.019 unit/mg of cell protein, respectively) compared to apoA-I_{WT} (0.095 ± 0.013 unit/mg of cell protein; Figure 7). Conversely, the K96C mutation had an approximate 30% reduction in arylesterase activity (0.067 ± 0.011 unit/mg of cell protein) compared to apoA-I_{WT}. Cys substitutions in the C-terminal portion of apoA-I, including

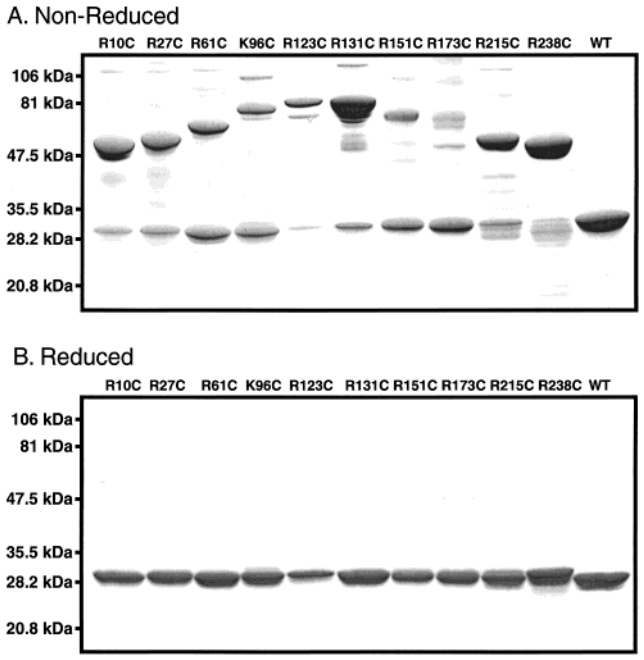

FIGURE 6: SDS−PAGE of recombinant apoA-I. Recombinant WT and Cys mutant apoA-I were examined by SDS−PAGE on 4−20% polyacrylamide gels. Human apoA-I-containing Cys substitution mutations are covalently linked dimers in nonreducing SDS−PAGE (panel A). The apparent molecular weights of the dimerized species differ in relation to the position of the Cys substitution. Under reducing conditions (5% β-mercaptoethanol), these proteins migrated as single bands of the same molecular weight as WT apoA-I (panel B). The gels were loaded with 5 μg of protein/lane.

apoA-I_{Paris} and apoA-I_{Milano} mutations, are not different from apoA-I_{WT} in their ability to elevate secreted PON arylesterase activity (0.086 ± 0.016 and 0.076 ± 0.029 unit/mg of cell protein, respectively) over conditioned medium without apoA-I (0.034 ± 0.010 unit/mg of cell protein). Inspection of the nonreduced gel profiles (Figure 6A) suggests that differences in PON activity cannot be attributed to the degree of dimerization of the Cys substitution mutants. The increase in PON activity in the N-terminal region is also not the result of disulfide bridge formation between PON and apoA-I, since no band corresponding to 70 kDa (expected molecular weight for the apoA-I/PON heterodimer) is observed by SDS−PAGE Western blot (Figure 3). Western blots probed for both apoA-I and PON showed no colocalization of the proteins (data not shown), corroborating this observation.

DISCUSSION

Previous work from our laboratory showed that apoA-I can recruit phospholipid and unesterified cholesterol from CHO cells to form unique subpopulations of nascent discoidal HDL particles (*24*). In this study, we developed a stably transfected CHO cell line expressing human serum PON to determine whether the assembly of nascent HDL was linked to the initial transport and lipoprotein association of secreted PON enzyme. We found that, rather than stimulating the extracellular accumulation of PON protein in conditioned medium, the recruitment of cellular lipid mediated by apoA-I greatly enhanced the specific activity of the PON associated with nascent HDL. Indeed, concentration dependence studies revealed that maximum lipidation of apoA-I was associated with full activation of PON enzyme. The observation that PON specific activity was dependent on the extent of apoA-I lipidation suggests that the extracellular assembly of nascent HDL may represent an important first step in the discrimination that PON exhibits for specific lipoprotein subclasses. This discrimination is exemplified by the in vivo observations that PON can be found associated with subclasses of HDL composed of apoA-I without apoA-II (*15*).

The observation that PON specific activity was maximized upon the lipidation of apoA-I in CHO cultures is consistent with the studies of Sorenson et al. (*16*) demonstrating that reconstituted apoA-I−phospholipid complexes increased PON specific activity. Moreover, deletion of PON's hydrophobic N-terminal domain was found to abrogate the association of PON with reconstituted HDL, leading to the conclusion that this region serves to anchor PON to HDL particles through, primarily, protein−phospholipid interactions (*16*). This interpretation, however, does not sufficiently address the exclusive affinity of PON with specific subpopulations of apoA-I containing HDL found in human plasma (*15*).

On the basis of the results of the present study, we would like to suggest an alternative hypothesis that evokes protein−protein interactions in the association of PON with apoA-I-containing HDL. We found that nascent HDL obtained from nontransfected CHO cells, as well as reconstituted apoA-I/EYPC complexes, produced only modest increases (30%) in PON specific activity compared to that (>200% increase) observed during the simultaneous incubation of apoA-I with transfected CHO cells. The latter reflects the incorporation of PON into nascent HDL during the assembly process mediated through the acquisition of cellular lipids by apoA-I. Although speculative, during HDL particle assembly PON may associate with the forming HDL in a series of interactions. It is known that, upon lipidation, apoA-I undergoes conformational changes detectable with secondary structure-specific monoclonal antibodies (*38*−*40*). Because the structure of lipid-free apoA-I is significantly different than that of lipidated apoA-I, it is reasonable to speculate that, during lipidation, regions of apoA-I become transiently available for PON interaction. The interaction of PON with these exposed regions may facilitate PON assembly onto HDL in a manner optimal for activation. This step may be bypassed when PON associates with preformed lipid complexes. Overall, it is likely that protein−protein interactions play a key role in directing the association of PON with the HDL particle.

The results of our cysteine-scanning mutagenesis studies provide additional evidence that protein−protein interactions between apoA-I and PON may play a role in optimizing PON activity during HDL assembly. Our rationale for these studies was initially based on the activity of serum PON in HDL deficiency states. It is known that a number of HDL deficiency syndromes are associated with dramatic reductions in serum PON protein and activity, with the exception of apoA-I_{Milano} (R173C), which does not appreciably alter PON activity (*19*). For this reason, we systematically created natural (R173C and R151C), as well as artificial, cysteine mutations within apoA-I primary sequence to determine whether specific regions of apoA-I were involved in supporting PON activity. The results of these studies indicate

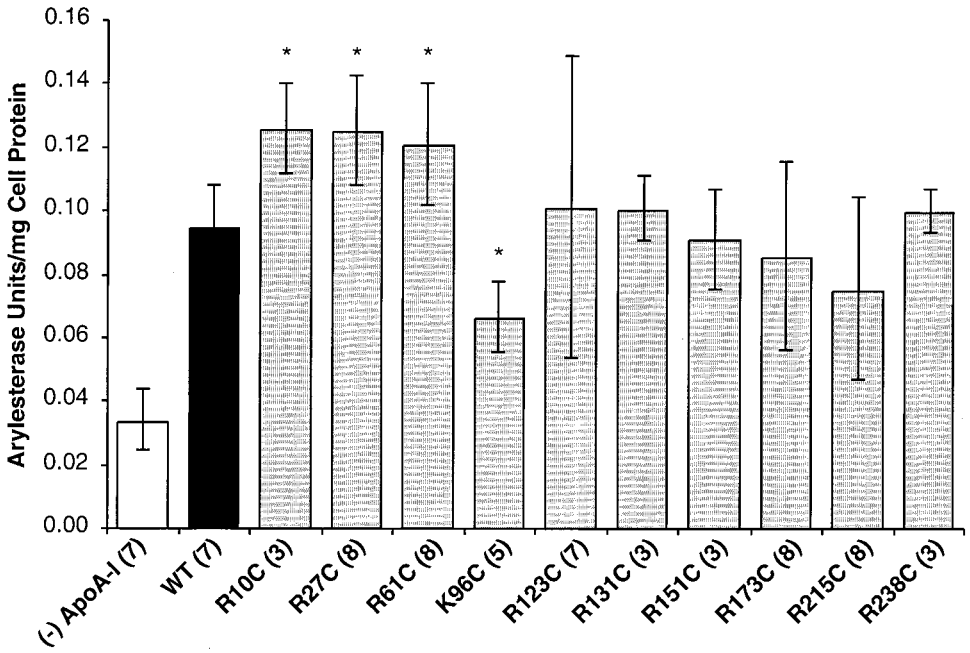

FIGURE 7: Effect of apoA-I mutations on PON activity. CHO cells expressing PON were cultured in the absence and presence of 10 μg/mL either WT or mutant apoA-I for 24 h, as described under Materials and Methods. Cys mutations in the N-terminal third of apoA-I (R10C, R27C, and R61C) elevated PON arylesterase activity significantly higher than WT apoA-I (0.126 $\pm$ 0.014, 0.125 $\pm$ 0.018, and 0.121 $\pm$ 0.019 versus 0.095 $\pm$ 0.013 unit/mg of cell protein, respectively) and a single Cys mutation was identified (K96C) with significantly lower activity (0.067 $\pm$ 0.011 unit/mg of cell protein) as determined by *t*-test analysis (*$p < 0.05$). The number of experimental repeats per mutant is noted in parentheses; experiments were carried out in triplicate.

that Cys substitutions within the C-terminal lipid-binding domains of apoA-I do not alter the specific activity of PON on nascent HDL, but rather, introduction of Cys residues within the N-terminal region of apoA-I produced significant changes in PON activity without affecting the mass of enzyme secreted from cells.

We identified a region in the N-terminal portion of apoA-I where the presence of cysteine appears to elevate PON activity (residues 10, 27, and 61) more effectively than apoA-I_{WT} and a second site (residue 96) where a cysteine substitution decreases apoA-I's ability to elevate PON activity. This ability to modulate PON activity is also assembly-dependent because nascent particles formed by the cysteine mutants when added to lipid-free PON show little increase in arylesterase activity (data not shown). A likely explanation to account for the altered PON activity in the mutant apoA-I nascent particles is that PON and apoA-I interact on the surface of the particles and that changes in the conformation of apoA-I's N-terminal portion affect the nature of this interaction. The specific nature of this interaction is the subject of further investigation.

We have presented evidence that lipid-free apoA-I does not alter PON activity, whereas the coassembly of PON onto apoA-I nascent particles substantially increases enzyme activity. We hypothesize that, during lipidation, apoA-I's structure is altered, exposing a latent PON interaction domain of apoA-I, directing PON to the HDL particle. It is likely that the nascent HDL provides PON with the proper lipid environment for enhanced enzyme activity. Furthermore, the Cys mutations suggest that the N-terminal region may be a critical domain in the proper presentation of PON's active site to the substrate, since mutations in this region were able to modulate PON activity.

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