Hydrolysis of platelet-activating factor by human serum paraoxonase

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Human serum paraoxonase (human PON1) has been shown to be important in the metabolism of phospholipid and cholesteryl ester hydroperoxides, thereby preventing the oxidation of low-density lipoprotein (LDL) and retarding atherogenesis. However, the exact substrate specificity of PON1 has not been established. In the present study we show that purified PON1 hydrolyses platelet-activating factor (PAF). We could find no evidence for contamination of our preparation with authentic platelet-activating-factor acetylhydrolase (PAFAH) by immunoblotting with a PAFAH monoclonal antibody or by sequencing the purified protein. In addition the specific PAFAH inhibitor SB-222657 did not affect the ability of PON1 to hydrolyse PAF ($30.1 \pm 2.8 \mu\text{mol/min per mg}$ of protein with no inhibitor;

$31.4 \pm 2.2 \mu\text{mol/min per mg}$ of protein with 100 nM inhibitor) or phenyl acetate (242.6 ± 30.8 versus $240.8 \pm 31.5 \mu\text{mol/min per mg}$ of protein with and without inhibitor respectively). SB-222657 was also unable to inhibit PAF hydrolysis by isolated human high-density lipoprotein (HDL), but completely abolished the activity of human LDL. Ostrich (*Struthio camelus*) HDL, which does not contain PON1, was unable to hydrolyse PAF. These data provide evidence that PON1 may limit the action of this bioactive pro-inflammatory phospholipid.

Key words: PON1, PAF acetylhydrolase, atherosclerosis, inflammation.

INTRODUCTION

At present, evidence is accumulating that high-density-lipoprotein (HDL) particles are involved in the protection of low-density lipoprotein (LDL) against oxidative modification. This mechanism appears to involve the hydrolysis of LDL-associated phospholipid and cholesteryl ester hydroperoxides and involves some of the enzymes located on HDL, including serum paraoxonase (PON1; EC 3.1.8.1) [1,2] and platelet-activating-factor acetylhydrolase (PAFAH; EC 3.1.1.47) [3,4]. Both enzymes are able to retard the oxidation of LDL by preventing the generation of lipid hydroperoxides, although the mechanisms involved are unclear. According to Watson et al. [5], oxidized phospholipids with long-chain fatty-acyl residues are hydrolysed by PON1, whereas PAFAH hydrolyses short-chain derivatives.

Human plasma PAFAH inactivates the bioactive phospholipid platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), therefore regulating its pathophysiological effects [6]. At physiological pH, about 70 % of the human plasma PAFAH is associated with LDL, the remainder being associated with HDL [7], although it has been suggested that an interchange occurs between the two lipoproteins. The role of PAFAH in lipid peroxidation is confusing, as evidence suggests that its action in hydrolysing lipid peroxides may lead to the formation of pro-atherogenic products [8]. However, other evidence suggests that the hydrolysis of oxidized phospholipids by PAFAH is anti-inflammatory [4,9]. At this time, it is not known whether both LDL- and HDL-associated PAFAH activities are mediated by the same enzyme. While human plasma LDL-associated PAFAH has been previously purified and biochemically characterized [10,11], the properties of HDL-associated PAFAH are still unknown.

In PON1 knock-out mice the HDL-associated PON1 is absent, but PAFAH remains the same as in wild-type mice and lecithin (phosphatidylcholine):cholesterol acyltransferase (LCAT) is

decreased by 25 %. HDL from the PON1 knock-out mice is pro-inflammatory, indicating the importance of PON1 in the anti-oxidative action of HDL and that PAFAH and LCAT are unable to prevent LDL oxidation in the absence of PON1 [12].

Recently it has been reported that the arylesterase/PON activities of PON1 and the hydrolysis of lipid peroxides involve two different active sites on the enzyme molecule [13,14]. The molecular structure of PON1 is very similar to that reported for human plasma PAFAH. Both proteins are glycoproteins with approx. 16 % N-linked carbohydrate [11,15] and an apparent molecular mass of 43–47 kDa [10,15]. The N-terminal regions have a high concentration of hydrophobic residues that may act as an anchoring point for lipoprotein complexes [11,16]. The high structural similarity between these two activities could account for the difficulty in separating PAFAH and PON1 using routine methods of purification [17]. In the present study we purified PON1 to homogeneity. We report for the first time that the purified protein exhibits both PON1 activity and PAFAH activity.

MATERIALS AND METHODS

Chemicals

[³H]PAF was purchased from DuPont NEN; the Bakerbond spe* Octadecyl (C₁₈) disposable extraction columns were from J. T. Baker (Phillipsburg, NJ, U.S.A.). Acrylamide/bisacrylamide 12 % ready-made gels were purchased from Bio-Rad (Richmond, CA, U.S.A.). Protein A-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Bicinchnonic acid (BCA) protein assay reagent and Coomassie Plus protein assay reagent were purchased from Pierce and Warriner (Chester, U.K.). The PAFAH inhibitor SB-222657 (an azetidinone compound) was a gift from SmithKline Beecham Pharmaceuticals (Harlow, Essex, U.K.). All other reagents were from Sigma (London) Chemical Co., Poole, Dorset, U.K., and were of analytical-reagent grade.

Abbreviations used: PON, paraoxonase; PON1, serum paraoxonase; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PAFAH, PAF acetylhydrolase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LCAT, lecithin (phosphatidylcholine):cholesterol acyltransferase; Con A, concanavalin A; BCA, bicinchoninic acid.

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Antibodies

Mouse monoclonal antibodies against LDL PAFAH were kindly given by SmithKline Beecham Pharmaceuticals and were used at a dilution of 1:2500. Rabbit polyclonal antibody to human PON1 was affinity-purified using Protein A–Sepharose and used at a dilution of 1:5000. Details of the specificity of this antibody have been described previously [18]. Anti-(rabbit IgG)–horseradish peroxidase conjugate and anti-(mouse IgG)–horseradish peroxidase conjugate were purchased from Sigma.

Rabbit anti-(rat PON) IgG was developed in one of our laboratories (Departamento de Medicina Legal Toxicología). Briefly, rabbits were immunized with rat liver PON purified by the method previously described by us [19]. The anti-(rat liver PON) antibody was purified from serum by Protein A–Sepharose affinity chromatography according to the manufacturer's instructions and used at a dilution of 1:2500.

Enzyme assays

PON activity towards paraoxon was quantified spectrophotometrically using 100 mM Tris/HCl buffer, pH 8, containing 2 mM CaCl_2 and 5.5 mM paraoxon. The reaction was monitored for 3 min at 25 °C by measuring the appearance of *p*-nitrophenol at 405 nm in an automated recording spectrophotometer (Beckman Du-68). All rates were determined in duplicate and corrected for non-enzymic hydrolysis [20]. Activity towards phenyl acetate was measured by adding serum or column fractions to 0.9 ml Tris/HCl buffer (100 mmol/l, pH 8.0) containing 2 mM CaCl_2 and 10 mM phenyl acetate. The rate of generation of phenol was determined at 270 nm, 25 °C, with the use of a continuously recording spectrophotometer (Beckman DU-68) [21]. PAFAH was determined by using 2-[acetyl- ^3H]PAF as substrate. Briefly, 0.1 mmol of substrate was incubated with human serum or fractions of column eluate in 0.1 M Hepes buffer, pH 7.2. After incubation, the reaction was stopped by adding 10 M acetic acid and 0.1 M sodium acetate. The [^3H]-acetate generated after *sn*-2-hydrolysis was separated from labelled substrate by solid-phase extraction chromatography and quantified by liquid-scintillation counting. Enzyme activity is expressed as $\mu\text{mol}/\text{min}$ per ml [7].

Preparation of lipoproteins

LDL (ρ 1.019–1.063 g/cm 3) and HDL (ρ 1.063–1.21 g/cm 3) were isolated by sequential ultracentrifugation using a Beckman L8-55M ultracentrifuge fitted with a Beckman 50.4 Ti Rotor (Beckman Instruments Ltd., Palo Alto, CA, U.S.A.) [22].

Buffers used in human PON1 purification

Buffer A was 25 mM Tris/HCl (pH 8.0)/1 mM CaCl_2 /5 μM EDTA. Buffer B was 25 mM Tris/HCl (pH 8.0)/1 mM CaCl_2 /5 μM EDTA, containing 4 M NaCl. Buffer C was 25 mM Tris/HCl (pH 8.0)/1 mM CaCl_2 /5 μM EDTA/20% (v/v) glycerol, containing 0.1% sodium deoxycholate. Buffer D was 50 mM Tris/HCl (pH 8.0)/10 mM CaCl_2 /10 μM EDTA/40% (v/v) glycerol/0.2% Emulgen 911. Buffer E was 25 mM Tris/HCl (pH 8.0)/5 mM CaCl_2 /5 μM EDTA/20% (v/v) glycerol/0.1% Emulgen 911. Buffer F was 25 mM Tris/HCl (pH 8.0)/5 mM CaCl_2 /5 μM EDTA/20% (v/v) glycerol/0.1% Emulgen 911, containing 0.35 M NaCl. Buffer G was 25 mM Tris/HCl (pH 8.0)/5 mM CaCl_2 /5 μM EDTA/20% (v/v) glycerol/0.1% Emulgen 911 containing 2 M NaCl. Buffer H was 25 mM Tris/HCl (pH 7.4)/1 mM CaCl_2 /0.15 M NaCl/0.1% (v/v) Emulgen 911. Buffer I was 25 mM Tris/HCl

(pH 7.4)/1 mM CaCl_2 /0.15 M NaCl/0.1% Emulgen 911 containing 0.35 M α -D-methyl mannopyranoside.

Purification of PON1

All purification procedures were carried out at room temperature unless otherwise stated. The purification was based on the method of Gan et al. [15].

Preparation of the serum

A 50 ml portion of serum was collected from a group of healthy, non-diabetic individuals who were homozygous for the PON1 QQ genotype. The serum was pooled and stored at –20 °C. Before use, the serum was thawed and assayed for total protein and PON1 and PAFAH activities. Thawed serum was gently mixed with 1 M CaCl_2 to give a final concentration of 10 mM and left for 30 min at room temperature. Any clot was removed by centrifugation (5000 g, 20 min) at 4 °C.

Cibacron Blue 3GA chromatography (non-specific affinity chromatography)

The serum was mixed with 100 ml of Cibacron Blue which had been pre-equilibrated in buffer B. The mixture was filtered, using suction, and the supernatant was measured for volume, protein concentration and PON1 and PAFAH activities before being discarded. The Blue Agarose was then washed five times with buffer B and twice with buffer A. Following the final wash, the dry Blue Agarose mixture was transferred to a chromatography column (33 cm $\times$ 2 cm). The gel was then washed with buffer A until the A_{280} decreased to less than 0.1. The bound enzyme was eluted from the column by using buffer C. Fractions (5 ml) were collected during the washing procedure and gradient elution. Fractions with the highest PON1 activity were used for DEAE-Sepharose chromatography.

DEAE-Sepharose CL-6B chromatography (ion-exchange chromatography)

The pooled fractions from the Cibacron Blue chromatography were diluted an in equal volume of buffer D to decrease the ionic strength and applied at 20 ml/h on to a column (33 cm $\times$ 2 cm; Pharmacia) of DEAE-Sepharose that had been pre-equilibrated with buffer E overnight. The column was first washed with the same buffer, and then the enzyme was eluted using a linear NaCl gradient from 0 to 0.35 M (buffers E and F respectively) at 0.5 ml/min. Fractions (5 ml each) were collected. The eluted fractions with the highest PON1 activity were pooled for the next step.

DEAE-Sepharose CL-6B chromatography (ion-exchange chromatography)

The pooled fractions from the first DEAE-Sepharose column were dialysed overnight against 2 litres of buffer E and applied at 0.5 ml/min on to a column (33 cm $\times$ 2 cm; Pharmacia) containing pre-equilibrated DEAE-Sepharose. After washing the column with buffer E, the bound enzyme was eluted by using a 300 ml linear salt gradient of NaCl (0–0.35 M) in buffer E at 0.5 ml/min. Fractions (5 ml each) were collected and those containing the highest PON1 activity were pooled for further purification.

Concanavalin A (Con A)–Sepharose chromatography (affinity chromatography)

The pooled fractions from the second DEAE-Sepharose column were dialysed overnight at 4 °C against 2 litres of buffer H to decrease the concentration of glycerol. All manipulations for this

purification step were performed at 4 °C. A suitable quantity of Con A was packed in a column (15 cm × 1 cm; Amicon Corporation) and equilibrated overnight with buffer H. The pooled fractions from the second DEAE-Sepharose chromatography step were applied at 0.35 ml/min on to the column. The column was washed with the same buffer and fractions (3 ml each) were collected. The bound enzyme was eluted with a linear gradient of 40 ml of buffer H and 40 ml of buffer containing 0.35 M methyl α -D-mannopyranoside (buffer I) at 0.5 ml/min. Fractions (1 ml each) were collected and those with the highest PON1 activity (54–57) were pooled (see Figure 4, peak 2, below) and concentrated by using a Centricon 30 microconcentrator (Amicon, Danvers, MA, U.S.A.). The Centricon concentrator was also used to remove most of the contaminating lectin (Con A) fragments from the pooled fractions.

Protein determination

The protein content of fractions from the column chromatographic separations was monitored by measuring A_{280} . Protein concentration was also estimated by the BCA method [23], on account of the high UV absorbance of Emulgen 911, using the BCA protein assay kit (Pierce and Warriner). The Coomassie Plus protein assay kit (also Pierce and Warriner) was used when the sample protein concentration was relatively low (1–50 μ g/ml). In both methods the standard curve was prepared from serial dilutions of BSA in water.

SDS/PAGE

Protein samples obtained during the different purification steps were analysed by SDS/PAGE [24], using a Bio-Rad Miniprotein II electrophoresis unit. The final monomer concentration was 12 % (w/v) for the separating gel and 4 % (w/v) for the stacking gel. Prior to loading, all samples were incubated in Laemmli sample buffer electrophoresis reagent (Sigma) containing of 4 % (w/v) SDS and 20 % (w/v) 2-mercaptoethanol for 2 min at 100 °C. The samples were run at a constant current of 20 mA, applied for 45 min. The proteins were silver-stained using the Bio-Rad Silver Stain Plus kit.

Western blotting

After SDS/PAGE the proteins were electrotransferred to a nitrocellulose membrane under a constant voltage of 10 V for 1 h. The membrane was then blocked with Tris-buffered saline (10 mM Tris/0.9 % NaCl, pH 7.4), containing 3 % BSA and 0.05 % Tween 20. The membrane was then incubated with 0.26 μ g/ml rabbit anti-(human serum PON1) IgG or 0.26 μ g/ml rabbit anti-(rat liver PON) IgG or 1.3 μ g/ml mouse anti-(LDL PAFAH). Subsequently the membrane was incubated with a specific secondary horseradish peroxidase-conjugate. PON1 and PAFAH bands were revealed in 0.3 % H_2O_2 with freshly prepared 3,3'-diaminobenzidine tetrahydrochloride as substrate.

Amino-acid-sequence analysis

For N-terminal sequencing, 50 μ g of protein from the Con A peak 2 was blotted on to a ProBlot™ membrane using a Pharmacia semi-dry blotter. After thorough washing, the protein on the membrane was directly sequenced using an Applied Biosystems ABI 473A pulsed liquid-phase protein sequencer according to the manufacturer's instructions.

Characterization of the purified enzyme

Michaelis constants were determined for the pooled concentrated fractions from the Con A column (peak 2), using PAF and paraoxon as substrates. The K_m was determined from Lineweaver–Burk plots using a weighted non-linear regression program (Enzfitter, Biosoft). The effects of a specific PAFAH inhibitor, SB-222657 [8], on PAF and paraoxon hydrolysis were also investigated in the pooled samples from the Con A column. The inhibition of PAF hydrolysis was studied by adding the effector (SB-222657 or EDTA) to the sample at the desired concentration and incubation at 37 °C for 10 min prior to the addition of [3 H]PAF. The residual enzyme activity was determined as described above. PON1 activity was determined by assaying the residual activity after preincubation of the Con A pooled fractions in presence of SB-222657, at the desired concentration, at 37 °C for 1 h. PAF hydrolysis and PON1 activity from the pooled Con A fractions were also determined in the presence of EDTA. The sample was incubated with 5 mM EDTA at 37 °C for 15 min before measuring PON1 activity and PAF hydrolysis.

RESULTS

Purification of PON1

Purification of PON1 from pooled plasma of QQ genotypes is summarized in Table 1. Essentially the same method as that used by Gan et al. [15] was employed, except that the DEAE ion-exchange-chromatography steps were changed to DEAE-Sepharose chromatography. The purification procedure involved four sequential steps: adsorption of the PON–HDL complex on to reactive Cibacron Blue–agarose, followed by sequential anion-exchange chromatography using two DEAE-Sepharose columns and a final affinity chromatography step using Con A–Sepharose, to remove any remaining albumin, and apolipoprotein A1.

The initial non-specific chromatography on Cibacron Blue was required to remove most of the albumin, which has a small amount of EDTA-resistant PON-like activity [15], and other soluble proteins. The fraction obtained after this treatment had a specific activity of 84.76 units/mg and showed an overall purification of 56-fold. Figure 1 shows the elution profile for this step. In the second step, the extract was chromatographed on DEAE-Sepharose CL-6B (Figure 2), which separates most of the PON activity from the apolipoproteins. This step gave a low yield (only 15 % of the initial activity applied), owing to the significant loss of active PON activity, but a relatively large amount of protein dissociated from the PON activity. During this step we detected the presence of two peaks containing both PON1 and PAFAH activities.

A second DEAE-Sepharose column purification step showed further improvements in specific activity (214.87 units/mg). The preparation was essentially free of albumin contamination. The elution profile of protein and PON1 and PAFAH activities were similar to those obtained in the first anion-exchange-chromatographic step (Figure 3).

After the second DEAE-Sepharose step, the preparation still contained significant amounts of apolipoprotein A1; an affinity-chromatographic step was introduced to purify further the human PON1. Con A binds structures containing an α -linked mannose residue [25] and is widely used in the purification of membrane and other glycoproteins [17]. The elution profiles of protein and PON1 and PAFAH activities from the Con A column are shown in Figure 4. This step produced two peaks, P1 and P2, with both PON1 and PAFAH activity. The first peak, P1, was eluted

Table 1 Summary of purification and yields of human PON1

One unit of arylesterase activity is 1 μ mol of substrate hydrolysed/min. Purification was measured as specific activity in purification step/specific activity in serum. The yield is the activity of fractions combined for the next purification step/total activity in serum $\times$ 100.

Step	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Overall purification (fold)	Overall yield (%)
Serum	50	38.30	1.51	5798.70	1	100
Cibacron Blue	31	23.44	84.76	1986.79	56.13	34.26
First DEAE-Sepharose	36	6.84	127.21	870.12	84.25	15.00
Second DEAE-Sepharose	17.5	2.98	214.87	640.33	142.30	11.04
Con A	4	0.748	266.74	199.52	176.65	3.44

Figure 1 Non-specific affinity chromatography on Cibacron Blue 3GA of human PON1

A 50 ml portion of serum from homozygotes for PON1 QQ were applied to a Cibacron Blue 3GA column. The material was eluted with buffer [25 mM Tris/HCl (pH 8.0)/1 mM CaCl_2 /5 μ M EDTA/20% (v/v) glycerol, containing 0.1% sodium deoxycholate]. Protein concentration was determined and fractions were assayed for PON activity using phenyl acetate as substrate. Abbreviation: U, units.

during washing of the column and its activity represented most of the PAFAH activity applied to the column. The retained material was eluted with a linear gradient of 0–0.35 M methyl α -D-mannopyranoside, yielding a second major peak (P2) with PON activity. The specific activity (266.74 units/mg) of the PON preparation was not improved from the previous step, probably owing to the significant loss of active PON due to the removal of phospholipids during the purification process [17]. After the Con A step the preparation was contaminated with some lectin fragments (Figure 5, lane B; 32 kDa), but these could be removed by using a Centricon 30 microconcentrator to wash the preparation. Con A peak 2 was chosen for further analysis because of its higher PON1 activity. No experiments were performed with peak 1.

The presence of calcium (as a cofactor) and Emulgen 911 (as detergent) in the buffers throughout the purification procedure was essential for maintaining the stability of the enzyme.

Purity of the preparation

Figure 5 illustrates the pattern of proteins found by SDS/PAGE after the Con A column. Lane B is a sample of purified human PON1 from the Con A step. The purified enzyme appears as two predominant bands of 49.4 kDa and 41.3 kDa, shown elsewhere

Figure 2 DEAE-Sepharose CL-6B chromatography of human PON1

Pooled fractions from the Cibacron Blue 3GA chromatographic step were applied to a DEAE-Sepharose CL-6B column. The active material was eluted by increasing the NaCl concentration. Protein concentration was determined and fractions were assayed for PON1 activity using phenyl acetate and PAFAH activity using PAF as substrate. Abbreviation: U, units.

to be PON1 monomeric species containing different linked sugar chains [15]. This result was confirmed by Western-blotting analysis using a mono-specific antibody raised against PON (results not shown). Purified PON has been determined in other studies to be a doublet within the 37–48 kDa-molecular-mass range [26]. The slowest-migrating band at 32 kDa corresponded to the lectin fragments from the Con A column [17].

Enzyme characterization

We determined the kinetic properties of the purified enzyme (Con A, peak 2) for two different substrates, namely PAF and paraoxon. Table 2 summarizes the K_m and V_{max} values for both substrates. The purified enzyme had higher affinity for PAF than for paraoxon and showed higher catalytic efficiency towards this substrate.

Western blotting

In order to determine whether PAF hydrolysis, which was still present after the last step of purification of PON1, was due to contamination with PAFAH protein or whether this PAF-hydrolysing activity was a previously unreported function of

Figure 3 Second DEAE-Sepharose CL-6B chromatography of human PON1

Fractions from the first DEAE-Sepharose CL6B column were pooled and dialysed as described in the Materials and methods section. This material was applied to a second DEAE-Sepharose CL6B column. The material was eluted by increasing the NaCl concentration. Protein concentration was determined and fractions were assayed for PON1 activity using phenyl acetate and for PFAH activity using PAF as substrate. Abbreviation: U, units.

Figure 4 Con A-Sepharose 4B purification of human PON1

Fractions from the second DEAE-Sepharose CL6B chromatographic step were pooled and dialysed as described in the Materials and methods section. This material was applied to a Con A column. The material was eluted by increasing the methyl α -D-mannopyranoside (α -DMMP) concentration. Two peaks of PON and PFAH activity (P1 and P2) were identified. Protein concentration was determined and fractions were assayed for PON1 activity using phenyl acetate and for PFAH activity using PAF as substrate. Abbreviation: U, units.

PON1, Western blotting was used. Following separation by SDS/PAGE, immunostaining confirmed the presence of PON1 protein (Figure 6A, lanes B, C and D) in both serum and the Con A pooled fractions at the expected molecular mass (49.3 kDa). As is shown in Figure 6(B), lane B, the antibodies to PFAH

Figure 5 SDS/PAGE of human PON1

The pooled fraction from the Con A column (P2) was analysed by SDS/PAGE and revealed by silver staining. Experimental conditions were as described in the text. Lane A contained 60 μ g of various molecular-mass standards: 2-galactosidase (126 kDa), fructose-6-phosphate kinase (102 kDa), pyruvate kinase (81 kDa), ovalbumin (53 kDa), lactate dehydrogenase (37.1 kDa) and triosephosphate isomerase (31.4 kDa). Lane B contained 200 μ g of Con A pooled sample (P2) with PON activity, being contaminated with Con A lectin fragments that are seen as a band at $\approx$ 32 kDa.

Table 2 Michaelis–Menten constants of purified PON1 for paraoxon and PAF

The K_m of human serum PON was determined using paraoxon and PAF. The conditions were as described in the Materials and methods section. Michaelis constants were determined from the Lineweaver–Burk plot. V_{max}/K_m is a measure of catalytic efficiency.

Parameter	Substrate ...	Paraoxon	PAF
K_m (mM)		1.38	0.0218
V_{max} (units/ml)		0.032	0.38
V_{max}/K_m		0.023	17.43

recognized PFAH in serum but not in the purified sample (lanes C and D).

Effect of inhibitors

The specific PFAH inhibitor SB-222657 did not inhibit either PAF or paraoxon hydrolysis in fraction P2 from the Con A column. EDTA, on the other hand, inhibited paraoxon hydrolysis but not the hydrolysis of PAF, consistent with there being two PON active sites, one Ca^{2+} -dependent and one not [13,14] (Table 3).

SB-222657 totally inhibited PAF hydrolysis by isolated human LDL (Table 4), but had no effect on the ability of isolated human HDL to hydrolyse PAF. HDL isolated from ostrich (*Struthio camelus*) serum, which is known not to have any PON1, was unable to hydrolyse PAF.

Amino-acid-sequence analysis

Amino-acid-sequence analysis of Con A peak P2 indicated that the 25 N-terminal amino acid sequence was that of PON1. No contaminating sequences were found (results not shown).

Cross-reactivity with anti-(rat liver PON1) IgG

The reactivity of anti-(rat liver PON1) towards PON1 in human serum and HDL and in pooled samples from the Con A column (P2) was analysed.

The immunoblot result (Figure 7) indicated that polyclonal IgG raised to rat liver PON1 had cross-reactivity with PON1

Figure 6 Western-blot analysis of human PON1 (A) and PAFAH (B) of P1 and P2 eluates of the Con A column

(A) The peak fractions P1 and P2 from the Con A column were subjected to Western-blot analysis using anti-PON antiserum (1:5000 dilution). Lane A contained 60 μ g of molecular-mass markers, lane B contained 14 μ g of human serum protein, lane C contained 20 μ g of P1 eluate and lane D contained 20 μ g of P2 eluate of the Con A column. (B) P1, P2 and serum were subjected to Western-blot analysis using a 1:2500 dilution of anti-PAFAH antiserum. Lanes are the same as those in (A).

Table 3 Effect of PAFAH and PON1 inhibitors on the ability of Con A peak 2 to hydrolyse PAF and phenyl acetate

All analyses were performed in triplicate.

Inhibitor	Substrate ...	Hydrolysis (μ mol/min per mg of protein)	
		PAF	Phenyl acetate
None		30.1 $\pm$ 2.8	240.8 $\pm$ 31.5
SB-222657			
10 nM		29.9 $\pm$ 3.1	242.7 $\pm$ 25.3
100 nM		31.4 $\pm$ 2.2	242.6 $\pm$ 30.8
EDTA (5 mM)		28.7 $\pm$ 4.2	0

Table 4 Effect of SB-222657 on PAF hydrolysis by isolated lipoproteins

Experimental details are given in the Materials and methods section. Each analysis was performed in triplicate.

	PAF hydrolysis (μ mol/min per mg of protein)	
	— SB-222657	+ SB-222657 (10 nM)
Human LDL	0.120 $\pm$ 0.037	0
Human HDL	0.078 $\pm$ 0.002	0.071 $\pm$ 0.002
Ostrich HDL	0	0

from human serum, showing an immunostaining band at 49.3 kDa in serum (lane B), purified sample (lane C) and HDL (lane D).

Figure 7 Cross-reactivity of anti-(rat liver PON) serum with human PON1

Immunostaining showing the cross-immunoreactivity of polyclonal antibody to rat liver PON against PON1 from human serum established by the presence of an immunostaining band at 53.5 kDa. Human serum, HDL and the pooled fractions with PON activity from the Con A column were subjected to Western-blot analysis using anti-(rat liver PON) serum (0.5 μ g/ml). Lane A contained 60 μ g of molecular-mass markers, lane B contained 14 μ g of human serum protein, lane C contained 15 μ g of Con A (P2) eluate and lane D contained 15 μ g of human HDL.

DISCUSSION

In the present study, we have provided evidence that PON1 purified from human serum can hydrolyse PAF and is responsible for the ability of HDL to hydrolyse PAF. Using an anti-(human LDL PAFAH) monoclonal antibody, selective PAFAH inhibitors and sequencing, we could find no evidence of PAFAH contamination of our purified PON1 preparation. Previous studies of PAFAH have largely been conducted using the LDL-associated enzyme, and no detailed information was available regarding the HDL-associated activity. It has been assumed that LDL and HDL PAFAH activities were attributable to the same enzyme, which could interchange between the two lipoproteins [7]. However, when macrophages (the major source of plasma PAFAH) were incubated with plasma depleted of PAFAH by inhibition with di-isopropyl fluorophosphate and then plasma lipoproteins re-isolated by ultracentrifugation, all the newly synthesized PAFAH was associated with lower-density lipoproteins, none being associated with HDL [27]. Also when proteins from HDL prepared by ultracentrifugation were separated by SDS/PAGE and the 25 N-terminal amino acids of each protein band were sequenced, we could find no evidence for the presence of authentic PAFAH (EC 3.1.1.47) on HDL (results not shown), indicating that PON1 is responsible for the hydrolysis of PAF by HDL. This finding may also explain the contaminating protein capable of hydrolysing PAF which was found by Akiyama and colleagues [28] when they were attempting to purify PAFAH from serum, which differed in sequence from the previously cloned LDL PAFAH [29].

Both PON1 and PAFAH have been shown to be protective against the development of atherosclerosis by hydrolysing, primarily, oxidized phospholipids generated on LDL [1–5]. However, studies using PON1 knock-out mice have indicated that PAFAH is unable to perform this function in the absence of PON1 [12], indicating that PON1 is the primary enzyme responsible for this protection.

PAF is a potent inflammatory mediator [6,7], and the primary function of PAFAH is to limit the action of PAF by hydrolysis to lyso-PAF, which is unable to interact with the PAF receptor. Therefore the hydrolysis of PAF by PON1 would be expected to result in the same effect. Thus, in addition to the well-known ability of PON1 to hydrolyse organophosphate insecticides and nerve gases, aryl esters and carbamates, plus the newly discovered

activities in hydrolysing glucocorticoid γ -lactone and cyclic carbonate pro-drugs [30] and homocysteine thiolactone [31], PON1 also has potent anti-inflammatory functions via the hydrolysis of oxidized phospholipids and PAF, which are potent pro-inflammatory molecules in blood-vessel walls. Such important physiological functions of PON1 are indicated by the high degree of structural conservation of PON1 proteins between mammalian species, illustrated here by the cross-reactivity of antibodies raised against rat liver PON towards human serum PON1, indicating the conservation of evolutionarily important functions such as the potent anti-inflammatory action of the enzyme.

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