

Human Serum Paraoxonases (PON1) Q and R Selectively Decrease Lipid Peroxides in Human Coronary and Carotid Atherosclerotic Lesions

PON1 Esterase and Peroxidase-Like Activities

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Background—Human serum paraoxonase (PON1) exists in two polymorphic forms: one that differs in the amino acid at position 192 (glutamine and arginine, Q and R, respectively) and the second one that differs in the amino acid at position 55 (methionine and leucine, M and L, respectively). PON1 protects LDL from oxidation, and during LDL oxidation, PON1 is inactivated.

Methods and Results—The present study compared PON1 isoforms Q and R for their effect on lipid peroxide content in human coronary and carotid lesions. After 24 hours of incubation with PON1Q or PON1R (10 arylesterase units/mL), lipid peroxides content in both coronary and carotid lesion homogenates (0.1 g/mL) was reduced up to 27% and 16%, respectively. The above incubation was associated with inactivation of PON1Q and PON1R by 15% and 45%, respectively. Lesion cholesteryl linoleate hydroperoxides and cholesteryl linoleate hydroxides were hydrolyzed by PON1 to yield linoleic acid hydroperoxides and linoleic acid hydroxides. Furthermore, lesion and pure linoleic acid hydroperoxides were reduced to yield linoleic acid hydroxides. These results thus indicate that PON1 demonstrates esterase-like and peroxidase-like activities. Recombinant PON1 mutants in which the PON1-free sulfhydryl group at cysteine-284 was replaced with either alanine or serine were no longer able to reduce lipid peroxide content in carotid lesions.

Conclusions—We conclude that PON1 may be antiatherogenic because it hydrolyzes lipid peroxides in human atherosclerotic lesions. (*Circulation*. 2000;101:2510-2517.)

Key Words: fatty acids ■ arteries ■ carotid arteries ■ atherosclerosis ■ lipids ■ lesion ■ cholesterol

Atherosclerosis, the major cause of morbidity and death in the Western world, involves complicated interactions among cells of the arterial wall, blood cells, and plasma lipoproteins.¹ Oxidative modification of LDL is a key event during early atherogenesis that contributes to cholesterol and oxysterol accumulation in the arterial wall and to lesion development.²⁻⁴ Oxidized lipids are found in atherosclerotic lesions and were shown to be related to the progression of atherogenesis.^{5,6} Paraoxonase (PON1) is associated in human serum with HDL.^{7,8} PON1 arylesterase/paraoxonase activities were shown to be inversely related to the risk of coronary heart diseases, hypercholesterolemia, and diabetes.^{9,10} During the development of atherosclerosis, PON1 accumulates in the arterial wall.¹¹ Recent studies have demonstrated that mice lacking serum paraoxonase were more prone to develop

atherosclerosis.¹² In these mice, both HDL and LDL were more susceptible to oxidation in comparison to control mice.¹²

PON1 contains 2 major polymorphisms as the result of amino acid substitution at position 55 (leucine vs methionine) and at position 192 (glutamine vs arginine),^{13,14} and these genetic polymorphisms have been suggested to be an independent risk factor for coronary artery disease.¹⁵

PON1 protects both LDL and HDL from oxidation,¹⁶⁻¹⁹ and recently we have demonstrated increased protection of PON1Q in comparison to PON1R against LDL oxidation.²⁰ This protection against LDL oxidation is probably related to the ability of PON1 to hydrolyze specific oxidized phospholipids^{16,19} and cholesteryl linoleate hydroperoxides¹⁷ in oxidized LDL.

Received September 17, 1999; revision received November 23, 1999; accepted December 12, 1999.

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The ability of PON1 to protect LDL against oxidation is paralleled by inactivation of the enzyme arylesterase activity.²¹ This PON1 inactivation results from an interaction of oxidized LDL-associated oxidized phospholipids and oxidized cholesteryl ester with the PON-free sulfhydryl group.²¹

It is not known whether PON1 can act on oxidized lipids in the atherosclerotic lesions as it does on lipoprotein-associated lipid peroxides. The present study thus analyzed the effect of PON1 on lipid peroxides in human atherosclerotic lesions derived from carotid or coronary arteries. We also compared the 2 polymorphic forms (PON1Q and PON1R) in relation to their effects on lesion lipid peroxides content and their inactivation characteristics during PON1 incubation with the lesions.

Methods

Coronary and Carotid Artery Lesions

Coronary Lesions

Patients undergoing coronary artery bypass surgery underwent additional coronary artery endarterectomy. In the process of construction of the proximal venous graft anastomosis of each patient, holes were punched in the ascending aortic walls. These pieces, 4.5 mm in diameter, were removed and served as control.

Carotid Lesions

Carotid atherosclerotic lesions were obtained from patients undergoing carotid endarterectomy for severe carotid stenosis >70%. These patients were either symptomatic or without symptoms; they underwent surgery under regional anesthesia. Complete atherosclerotic plaques were removed, including the common internal and external carotid parts of the plaque. The plaques were immediately placed in saline. Lesions were washed in saline, dried, and their weight measured. The coronary and carotid lesions were cut into small pieces and rinsed in PBS, followed by their sonication in an ultrasonic processor (3×20 seconds at 80 W). The homogenates were diluted in PBS to a concentration of 0.1 g/mL of dry weight.

Serum PON1 Purification

PON1 was purified from the sera of healthy human volunteers previously identified as homozygous for PON1Q or for PON1R. Phenotyping was done as previously described.²² To purify PON1, 1 mol/L of CaCl₂ (50 mL) was added per liter of serum and centrifuged at 8000g for 30 minutes at 4°C to remove the fibrin clot. The supernatant was mixed with Blue Agarose (Cibacron Blue 3 GA, Agarose, Type 3000, Sigma Chemical Co) in a solution containing 3 mol/L NaCl, 50 mmol/L Tris/HCl, pH 8.0, with 1 mmol/L CaCl₂ and 5 mmol/L EDTA.^{22,23} PON1 was eluted with 0.1% deoxycholate. The Blue Agarose-eluted PON1 (Figure 1A) was further purified by DEAE Bio gel (Bio-Rad Laboratories) anion exchange chromatography with the use of a NaCl linear gradient, as previously described (Figure 1B, Reference 23). This was followed by a second DEAE chromatography (Figure 1C), in which the nonionic detergent tergitol (NP-10, Sigma Chemical Co) replaced Emulgen 911 (KaO Corp). To remove residual contamination of albumin, LCAT, and PAF-AH, Con-A column was used with a linear gradient (0 to 0.15 mol/L) of methyl- α -D-manno-pyranoside (Figure 1D). Protein Con-A fragments were removed from PON1 by Centricon 100 microconcentrators (Amicon Inc). The purity of the enzyme was verified by SDS-PAGE (Figure 1). This preparation of PON1 was pure to homogeneity and free of any contamination of apolipoprotein A-I that could possibly contribute to hydrolytic effects on lipid peroxides.²⁴ The pure PON1 preparation did not react with antibodies toward apolipoprotein A-I, LCAT, and PAF-AH. Figure 1 demonstrates the major purification steps of human serum paraoxonase up to the final preparation, which was pure to homogeneity. Purified PON1 was kept at 4°C in 25 mmol/L Tris-HCl buffer,

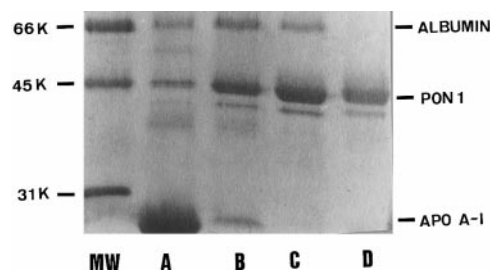


Figure 1. SDS-PAGE analysis of human PON1 purification. First lane on left represents low-molecular-weight (MW) standards (31K, carbonic anhydrase; 45K, ovalbumin; 66K, bovine serum albumin). A, Blue agarose; B, first DEAE; C, second DEAE; D, Con-A, methyl-manno-pyranoside gradient. Note that PON1 has 2 bands (glycosylated forms) and final PON1 preparation used in the present study (D) is pure with regard to homogeneity and free of apolipoprotein A-I (APO A-I) and albumin. Detailed purification steps are described in Methods.

1 mmol/L CaCl₂, 20% glycerol, 0.1% tergitol, pH 8.0, to preserve its activity.

Paraoxonase Arylesterase Activity

Arylesterase activity was determined spectrophotometrically at 270 nm with phenyl acetate used as the substrate. The assay mixture included 1.0 mmol/L of phenyl acetate and 0.9 mmol/L CaCl₂ in 20 mmol/L Tris HCl, pH 8.0, at 25°C. Nonenzymatic hydrolysis of phenyl acetate was subtracted from the total rate of hydrolysis. The E₂₇₀ for the reaction is 1310 mol/L⁻¹ · cm⁻¹ and 1 unit of arylesterase activity is equal to 1 micromole of phenyl acetate hydrolyzed per milliliter per minute.²³

Site-Directed Mutagenesis, Transfection, and Expression of Recombinants

The procedures for the production of recombinant PON1s (ie, production of wild-type PON1Q as well as mutants with alanine or serine in place of cysteine-284) has been described in detail elsewhere.²⁵ As a control, we used Chinese hamster ovary-K1 cells transfected with the plasmid glutamine synthetase expression vector alone, with no PON1 cDNA insert. Incubation of lesions with recombinant PON1s were carried in 1 mL of Ultra Culture (Bio Whittaker) media containing equal activities of the various PON1 preparations (0.3 arylesterase units/mL).

Lesion Oxidized Lipids

Lipid Peroxides

The amount of lipid peroxides in lesion samples before and after their incubation with PON1 was determined by the method of El-Saadani et al.²⁶ The presence of detergent in the PON1 samples did not significantly affect the decrement in lesion lipid peroxides as observed after incubation of lesion specimens with PON1 samples prepared with or without detergent (data not shown).

Synthesis and Analyses of Cholesteryl Linoleate Hydroperoxide, Cholesteryl Linoleate Hydroxide, Linoleic Acid Hydroperoxide, and Linoleic Acid Hydroxide

Cholesteryl linoleate hydroperoxide (CL-OOH) was prepared by oxidation with *t*-butyl hydroperoxide.²⁷ CL-OOH, cholesteryl linoleate hydroxide (CL-OH), and cholesterol linoleate (CL) were determined by high-performance liquid chromatography (HPLC, Varian, equipped with C-18 column, 25-cm length, 0.4-cm diameter, 5- μ m particle size). CL-OOH and CL-OH were detected at 234 nm; CL was detected at 210 nm. Methanol/water was used as eluent, at a flow rate of 1.0 mL/min.²⁸ Linoleic acid hydroperoxide (L-OOH) was synthesized from 50 mg of linoleic acid by oxidation with *t*-butyl hydroperoxide.²⁷ Both lipid hydroperoxides, CL-OOH and L-OOH, were reduced to CL-OH and linoleic acid hydroxide

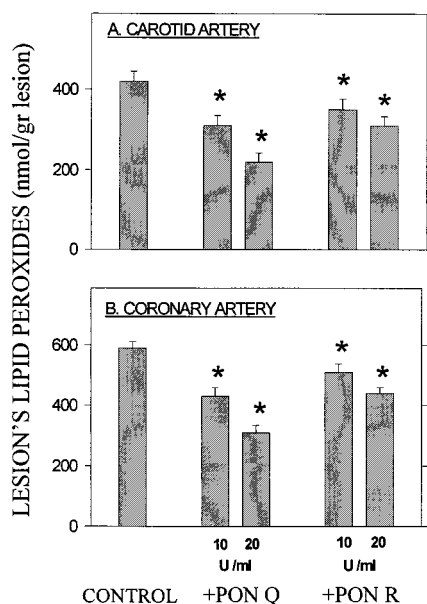


Figure 2. Effect of PON1Q and PON1R on human lesion lipid peroxide content: concentration study. Lesion homogenates (0.1 g in 1 mL) derived from carotid artery (A) or from coronary artery (B) specimens were incubated without (control) or with PON1Q or PON1R (10 or 20 arylesterase units/mL) for 24 hours at 25°C. Lesion lipid peroxide content was measured at the end of the incubation period. Results are given as mean \pm SD ($n=5$). * $P<0.01$ vs control.

(L-OH), respectively, by the same method.²⁷ CL-OOH (8 mg) was dissolved in ethanol (1 mL), and excess of sodium borohydride was added. The reaction mixture was stirred for 30 minutes at 4°C and then stopped by the addition of 5 mL ether and 1 mL of water, containing 10 mg of citric acid. The organic phase was then separated and washed twice with distilled water, dried, and evaporated. Then, the dried sample was dissolved in acetone and injected (20 μ L) into the HPLC. The detector was monitored at 210 nm for linoleic acid and at 234 nm for L-OOH and for L-OH. The eluents were a mixture of water/acetonitrile/tetrahydrofuran/acetic acid (volume ratios of 40:40:20:0.25) at a flow rate 1.0 mL/min.

Statistical Analyses

The Student's *t* test was used to analyze the significance of the results. In some cases (Figures 2 and 6), ANOVA was used followed by the post hoc Tukey's test for equal size samples. Results are given as mean \pm SD.

Results

Selective Reduction of Lipid Peroxides in Human Carotid and Coronary Lesions by PON1Q Versus PON1R

PON1 activity could be demonstrated in both carotid and coronary lesion specimens. In carotid and coronary lesions, 1.80 ± 0.12 and 2.10 ± 0.15 arylesterase units/g lesion were detected, respectively ($n=6$), whereas in nonlesion arterial specimens, PON1 arylesterase activity was only 0.86 ± 0.05 units/g lesion ($n=6$).

It was previously demonstrated that PON1Q allozyme possesses a greater ability than PON1R allozyme in protecting LDL lipids from oxidation.^{20,29,30} Thus, we analyzed the ability of both PON1 allozymes to reduce atherosclerotic lesion oxidized lipids from carotid or coronary arteries. Incubation of PON1Q (10 arylesterase units/mL) with the

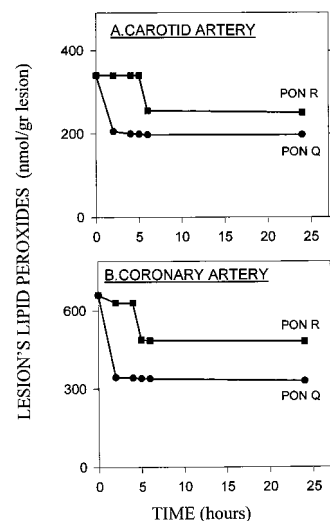


Figure 3. Effect of PON1Q and PON1R on human lesion lipid peroxide content: time study. Lesion homogenates (0.1 g in 1 mL) derived from carotid artery (A) or from coronary artery (B) specimens were incubated with PON1Q or PON1R (20 arylesterase units/mL) for up to 24 hours at 25°C. Lipid peroxides were measured every hour during first 6 hours and after 24 hours of incubation. Results represent mean \pm SD of 3 different experiments.

atherosclerotic lesions for 24 hours at 25°C resulted in a decrement in lipid peroxides in the carotid and the coronary arteries by 27% and 25%, respectively. PON1R, at a similar activity, caused only 16% and 14% reduction, respectively (Figure 2, A and B). A more substantial reduction in lesion lipid peroxide content, up to 44% or 26%, respectively, was obtained with the use of 20 arylesterase units of PON1Q or PON1R/mL, respectively (Figure 2, A and B). Similar results were obtained for both types of human lesions (carotid and coronary, Figure 2, A and B). PON1Q allozyme has a greater ability than PON1R allozyme to reduce lipid peroxide content in human atherosclerotic lesions. In control, nonlesion arterial specimens, lipid peroxide content was <50 nmol/g and PON1 had no effect on the lipid peroxide content (data not shown).

Kinetic analysis of the reduction in lesion lipid peroxide content during incubation with PON1Q or PON1R (20 arylesterase units/mL) revealed that the time required for the initiation of the decrement in lesion lipid peroxides was only 120 minutes for PON1Q and up to 300 minutes for PON1R. At these time points, maximal effects of PON1Q (39% reduction) or PON1R (25% reduction) on carotid lipid peroxide content were obtained (Figure 3A). Similar results were obtained after incubations of PON1Q or PON1R with coronary lesions (Figure 3B), with 48% or 26% reduction, respectively, in lesion lipid peroxides.

Mechanisms for Reduction of Lesion Lipid Peroxides by PON1

Cholesteryl linoleate, the major lipid moiety of native LDL, is extensively oxidized under oxidative stress to yield various types of lipid peroxides. Carotid lesions contain both CL-OOH and CL-OH (Figure 4A).

On incubation of carotid lesion homogenate (0.1 g/mL) with PON1Q (20 arylesterase units/mL) for 24 hours at 25°C,

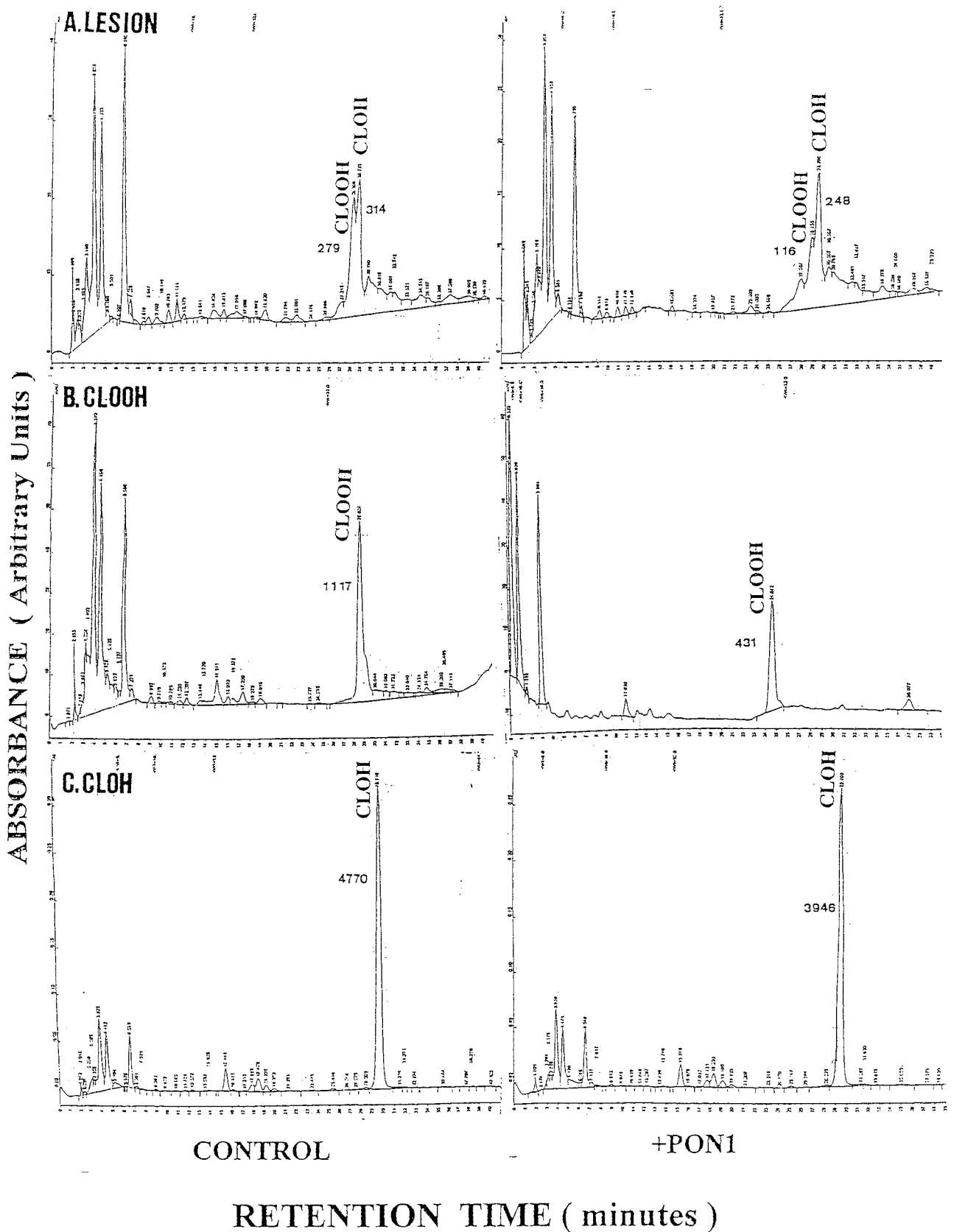


Figure 4. Effect of human serum paraoxonase (PON1) on carotid lesion, CL-OOH, and CL-OH. Carotid lesion (0.2 g homogenate/2 mL) (A), purified CL-OOH (50 μ g/mL, B), or purified CL-OH (50 μ g/mL, C) were incubated without (control) or with PON1Q (20 arylesterase units/mL) for 24 hours at 25°C. Then, all samples were extracted with hexane/isopropanol (3:2 vol/vol). Upper hexane phase was collected and dried under nitrogen. Dried samples were then dissolved in 60 μ L of ethanol and analyzed for CL-OOH and CL-OH content by HPLC. Numbers close to each peak represent peak area in arbitrary units ($\times 10^{-3}$). Profile represents 1 of 3 similar experiments.

a substantial decrement in lesion CL-OOH and CL-OH content by 58% and 21%, respectively, was observed (Figure 4A). Similar decrements were obtained when PON1Q (20 arylesterase units/mL) was incubated with 50 $\mu\text{g/mL}$ of purified CL-OOH (Figure 4B) or with 50 $\mu\text{g/mL}$ of purified CL-OH (Figure 4C) with reductions in the content of these compounds by 61% and 17%, respectively. The reduction in CL-OOH and in CL-OH content by PON1Q might be the result of PON1Q esterase-like and/or peroxidase-like activities. To question possible PON1 esterase activity on lesion CL-OOH and CL-OH, we measured the amount of L-OOH and L-OH after PON1 incubation with lesion or with purified CL-OOH or CL-OH (Figure 5). Carotid lesions contained almost no free L-OOH and L-OH, as demonstrated in Figure 5A. Incubation of lesion homogenate (0.1 g/mL) with PON1Q (20 arylesterase units/mL) resulted the production of L-OOH and L-OH (Figure 5A). Similar results were obtained on PON1Q incubation with purified CL-OOH (Figure 5B).

These results clearly suggest an esterase-like activity for PON1, as the decrement in lesion CL-OOH and CL-OH content was associated with the formation of L-OOH and L-OH (a cleavage of the ester bond between cholesterol and linoleic acid hydroperoxide/hydroxide). To question possible peroxidase-like activity of PON1Q, we incubated PON1Q (20 arylesterase units/mL) with 50 $\mu\text{g/mL}$ of purified L-OOH for 24 hours at 25°C. HPLC analysis of L-OOH and L-OH content demonstrates a 53% reduction in L-OOH content and the formation of L-OH (Figure 5C). These results suggest a peroxidase-like activity for PON1Q because it can reduce L-OOH to L-OH. This pattern was shown with the use of whole-lesion, purified CL-OOH or purified L-OOH (Figure 5). In contrast, PON1 did not act on nonoxidized cholesteryl linoleate. On incubation of PON1Q (20 arylesterase units/mL) with purified CL (50 $\mu\text{g/mL}$) for 24 hours at 25°C, the CL peak area $[(997 \pm 47) \cdot 10^{-3} \text{ arbitrary units, } n=3]$ was not significantly changed $[(965 \pm 58) \cdot 10^{-3} \text{ arbitrary units, } n=3]$.

PON1 contains only 1 free sulfhydryl group at cysteine-284. We have recently shown that recombinant PON1 mutants in which the free sulfhydryl group was replaced with either alanine or serine were no longer able to protect LDL against oxidation.²⁰ To address the possible role of the PON1 Cys-284 residue in PON1 hydrolytic action on lesion oxidized lipids, we used the above recombinant PON1Q mutants in which the cysteine residue at position 284 was replaced by either serine (Cys 284 Ser) or alanine (Cys 284 Ala) by site-directed mutagenesis (Figure 6). Whereas the recombinant PON1Q wild-type (WT) caused a significant 35% reduction in carotid lesion lipid peroxide content after 24 hours of incubation (as did the human serum paraoxonase), both mutants had no significant effect on lesion lipid peroxide content (Figure 6).

PON1 Inactivation During Incubation With Atherosclerotic Lesions

We have previously demonstrated that during LDL oxidation, a time-dependent inactivation of PON1 arylesterase activity by oxidized lipids in oxidized LDL was observed, with PON1R being more inactivated than PON1Q.²¹ In the present study, we analyzed PON1Q and PON1R arylesterase activi-

ties during PON1 incubation with coronary or carotid lesions. Incubation of PON1Q or PON1R (20 arylesterase units/mL) for up to 24 hours at 25°C revealed that PON1Q arylesterase activity was more resistant to inactivation than PON1R (Figure 7). The arylesterase activity of PON1Q decreased after 24 hours of incubation by only 17% and 15%, respectively, on carotid (Figure 7A) or coronary (Figure 7B) lesions. In contrast, PON1R lost as much as 38% and 32%, respectively, of its arylesterase activity after 24 hours of incubation with carotid (Figure 7A) or coronary lesions (Figure 7B).

Discussion

The present study demonstrated for the first time that human serum paraoxonase (PON1) has the capacity to reduce oxidized lipids in human atherosclerotic lesions derived from either carotid or coronary artery specimens. We have demonstrated that PON1Q was more potent than PON1R in this respect, as previously shown for their selective effects on LDL oxidation.^{20,29,30} On PON1 incubation with atherosclerotic lesions, PON1R arylesterase activity but not PON1Q activity was substantially decreased, as was also shown previously for their response to LDL oxidation.²¹ Thus, the better action (more rapid and to a higher extent) of PON1Q versus PON1R on lesion oxidized lipids may be related to the fact that it was better preserved during PON1 incubation with the lesions.

The present study further extended the previously shown protective effects of PON1 against LDL and HDL lipid peroxidation¹⁶⁻²⁰ to additional oxidized lipids—those found in atherosclerotic lesions. The protective role of PON1 against lipoprotein lipid peroxidation was suggested to be related to the ability of PON1 to hydrolyze oxidized lipids including oxidized phospholipids^{16,19} and cholesteryl ester hydroperoxides.¹⁷ In the present study, we have clearly demonstrated that the action of PON1Q on lesion cholesteryl ester hydroperoxides involves esterase-like and peroxidase-like activities. The lesion content of both CL-OOH and CL-OH was significantly reduced, and this was associated with the formation of the corresponding free fatty acids, thus demonstrating an esterase activity of PON1. PON1 peroxidase-like activity reduces L-OOH to L-OH and CL-OOH to CL-OH in whole lesions and in purified L-OOH or CL-OOH. PON1 used in the present study was free of any contaminated apolipoprotein A-I. Thus, the observed hydrolytic effects of PON1 on cholesteryl linoleate hydroperoxides and on cholesteryl linoleate hydroxides could not be related to apolipoprotein A-I.²⁴ Furthermore, we have shown that PON1 recombinant, which is free of apolipoprotein A-I, still significantly decreased lesion lipid peroxide content.

We have recently demonstrated that the PON1 active site for protection against LDL oxidation requires the PON1-free sulfhydryl group at cysteine-284.²⁰ The present study shows that for PON1 action on lesion lipid peroxides, cysteine-284 is also required. This site was recently shown to be responsible also for PON1 inactivation by oxidized lipids in oxidized LDL.²¹

Oxidized lipids in the atherosclerotic lesion have atherogenic properties.³¹ Compounds (from diet or drugs) with

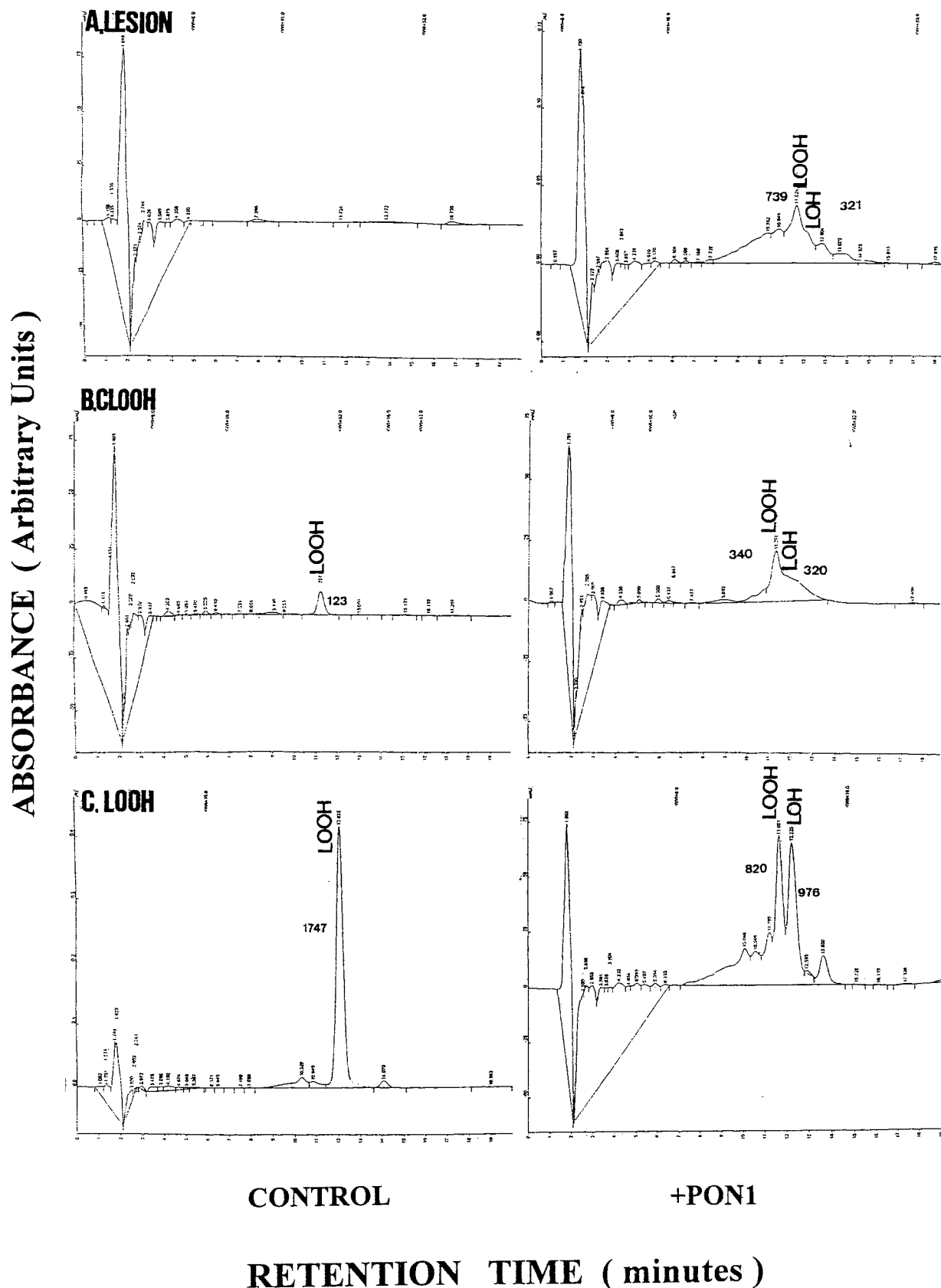


Figure 5. Effect of human serum paraoxonase (PON1) on formation of L-OOH and L-OH in carotid lesion. Carotid lesion (0.2 g/2 mL) homogenate (A), purified CL-OOH (50 μ g/mL, B), or purified L-OOH (50 μ g/mL, C) were incubated without (control) or with PON1Q (20 arylesterase units/mL) for 24 hours at 25°C. At end of the incubation period, all samples were extracted. Dried samples were dissolved in 60 μ L of ethanol and analyzed for L-OOH and L-OH content by HPLC. Numbers close to each peak represent peak area in arbitrary units ($\times 10^{-3}$). HPLC profile represents 1 of 3 similar experiments.

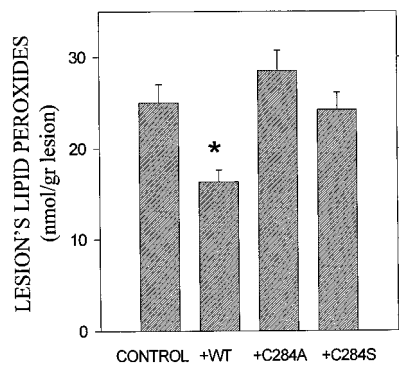


Figure 6. Effect of recombinant PON1Q cysteine mutants on carotid lesion lipid peroxide content. Carotid lesion homogenate (0.05 g in 0.5 mL) was incubated for 24 hours at 25°C without (control) or with 0.3 arylesterase units/mL of recombinant PON1Q wild-type (WT), PON1Q in which Cys-284 was replaced by serine (C284S), or by alanine (C284A). At end of the incubation period, lesion lipid peroxide content was determined. Results are given mean \pm SD. * P <0.01 vs control, n =3.

antioxidative activity against lipoprotein oxidation preserved PON1 activity, in association with their ability to protect lipoproteins against oxidation.^{21,32} The finding of active PON1 in the atherosclerotic lesion may suggest that in response to high oxidative stress in the lesion area, PON1 was delivered to such an area to hydrolyze and remove atherogenic lipid peroxides from the lesion. We conclude that the physiological role of human serum paraoxonase (PON1) might be to remove oxidized lipids from human atherosclerotic lesions, secondary to its hydrolytic (esterase-like and

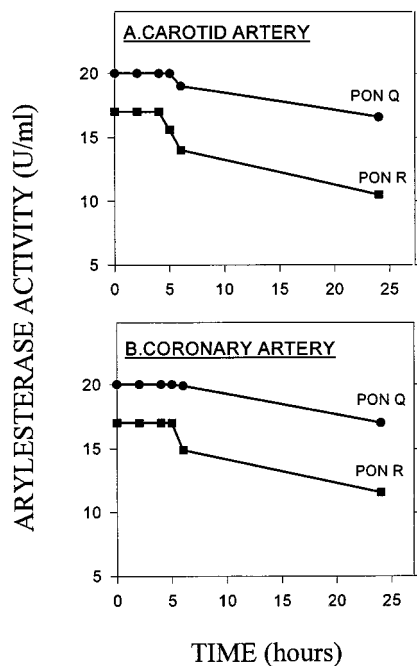


Figure 7. Inactivation of PON1Q and PON1R during incubation with human lesions. Lesion homogenates (0.2 g in 2 mL) derived from carotid (A) or coronary (B) artery specimens were incubated with PON1Q or PON1R (20 arylesterase units/mL) for up to 24 hours at 25°C. PON1 arylesterase activity was measured at various time points; every hour during the first 6 hours and after 24 hours of incubation. Results are given as mean \pm SD of 3 different experiments.

peroxidase-like activities) actions on specific lipid peroxides. Interventional means such as dietary antioxidants to preserve or even to increase PON1 activity may thus contribute to attenuation of atherogenesis.^{15,32}

Acknowledgment

This study was supported by a grant from the Rappaport Family Institute for Research in the Medical Sciences, Haifa, Israel.

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