Paraoxonase protection of LDL against peroxidation is independent of its esterase activity towards paraoxon and is unaffected by the Q→R genetic polymorphism

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Abstract **High density lipoprotein (HDL)-associated paraoxonase (PON) seems to play a major role in the protection of low density lipoprotein (LDL) against peroxidation by HDL, and the partly purified enzyme exerts a dose-dependent protective effect. A common polymorphism of the human gene (192 Q→R) modulates paraoxonase activity but purified enzyme from either genotype is equally effective against LDL peroxidation. The inhibition of Cu^{2+}-induced LDL peroxidation by HDL was monitored by lipid peroxide assay and change in LDL electrophoretic mobility. We show that HDL from type 2 diabetic patients with the QQ or RR genotype (n = 12 for each) reduce, to the same extent, both peroxide production (by 60.6 ± 20.0 and 63.9 ± 23.5%) and relative change in mobility (61.3 ± 21.8 and 61.4 ± 26.5%) despite a 6-fold difference in paraoxonase activity (47.4 ± 4.4 vs. 299.7 ± 23.7 U/l, $P < 0.0001$). Protection was, however, related to paraoxonase activity, but with a different efficiency in each group corresponding to a better protection per unit of enzyme in the QQ genotype group. Inactivation of PON activity by heating (56°C, 10 min) or by EDTA was totally without effect on protection, which remained correlated with the paraoxonase activity measured prior to inactivation. In summary, these results suggest that the protein bearing both paraoxonase and arylesterase activities also possesses a third thermostable property, closely associated with paraoxon hydrolysis activity and unaffected by PON genetic variability.—**Cao, H., A. Girard-Globa, F. Berthezene, and P. Moulin. **Paraoxonase protection of LDL against peroxidation is independent of its esterase activity towards paraoxon and is unaffected by the Q→R genetic polymorphism.** ***J. Lipid Res.*** **1999.** 40: **133–139.**

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Oxidation of LDL is considered an efficient atherogenic process (1–5), especially in the light of a recent demonstration of significant protection by antioxidant therapy (6). Aside from antioxidant agents that prevent the occurrence of oxidation, other mechanisms act by reducing early oxidative products and thereby suppressing the propagation of oxidation (7). One of these is thought to be paraoxonase (PON) (8), an HDL-bound ester hydrolase to which is attributed a significant part of the protective properties displayed by HDL against LDL oxidation (9–12). PON-containing HDL have proved capable of preventing the oxidative modification of LDL whether obtained by chemical interventions such as incubation in the presence of copper (9, 10) or by more biological means such as interaction with vascular cells (13, 14).

Paraoxon hydrolysis activity (PON p.ase) varies widely among individuals. Part of this variability finds it source in the polymorphism of PON1 gene (15). A Q→R substitution at position 192 is determinant for activity, the R allele coding for a protein that displays several-fold higher activity towards paraoxon hydrolysis than the Q allele (16, 17). An M→L substitution at position 55 has a lesser effect on activity, while a strong linkage disequilibrium exists between L-55 and A-192 (18). Arylesterase activity borne by the same protein (PON ar.ase) is not affected by either polymorphism and can be considered an index of actual protein concentrations, independent of PON1 variability. If, indeed, protection by PON of LDL against oxidation is a meaningful phenomenon, then the R allele should confer increased cardiovascular protection compared to Q. Recent studies performed on the basis of this hypothesis have, on the contrary, revealed that the RR genotype was more prevalent among subjects with a history of cardiovascular disease than in controls (19–22). These results were, however, quite controversial (23–25) and we have, ourselves, shown that this polymorphism of PON1 has no effect on the vascular pathology of diabetic patients as evaluated by intima-media thickness, a continuous variable (26).

This raises a number of questions: *1*) whether PON is at

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; PON, paraoxonase; AE, arylesterase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PON p.ase, paraxon hydrolysis activity of paraoxonase; PON ar.ase, arylesterase activity of paraoxonase.

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all involved in protection of LDL against oxidation; *2*) whether PON activity as measured by paraoxon hydrolysis actually reflects the anti-oxidant capacity of the enzyme; and *3*) whether the effect might not be that of some other HDL component. The answer seems to be that PON is indeed involved as purified preparations of the protein are capable of hydrolyzing oxidized palmitoyl-arachidonyl-phosphatidylcholine, a component of oxidized LDL (13) and of protecting HDL against oxidation (27). Both alloenzymes prove, however, equally efficient despite wide differences in PON p.ase although it is not excluded that an allotypic difference might exist when the enzyme is associated with HDL or when it is acting on nascent products. A recent study has reported a more lasting protective effect of HDL bearing the low PON p.ase Q type protein during prolonged oxidative incubation of LDL (28, 29). This has been interpreted as indicative of a superior protective capacity for the Q allozyme despite its lower activity. All three 192 genotypes displayed similar protective properties towards oxidation of homologous LDL after relatively short incubation times (4 h) during which generation of peroxides was surprisingly low, even in the absence of HDL. Moreover, the use in this experiment of homologous LDL, already conditioned by their own HDL, precluded interpretation of specific effects of the isoforms.

The uncertainties relative to the actual impact of PON1 genetic polymorphism on oxidative mechanisms have led us to re-address this potentially important point. Because of their propensity to cardiovascular accidents linked to low HDL more than to high LDL, type 2 diabetics are most likely to be affected by insufficient protection against oxidation. We have used HDL from type 2 diabetic patients homozygous for the 192 polymorphism and tested their protective capacity on strictly identical LDL, both before and after inactivation of PON p.ase by various means.

SUBJECTS AND METHODS

Twenty four subjects were selected for this study from a population of over 100 NIDDM patients on the basis of homozygous PON1 genotypes: half QQ and half RR. The study was approved by our local ethics committee. Informed consent was obtained from all.

The patients presented the characteristics of a moderately well controlled type 2 diabetes: mean HbA1c around 8%, overweight and mild hypertriglyceridemia (Table 1). There were no significant differences between QQ and RR genotypic groups for any of the clinical parameters. Both groups included men and women (all menopausal) in ratios of 1:3 and 1:1 respectively for AA and BB subgroups with no evidence of sex-linked differences in either.

Blood samples were drawn after an overnight fast: in dry tubes for HDL separation and measure of enzyme activity, on EDTA for LDL preparation and DNA extraction from cells.

DNA analysis

PON1 was genotyped by restriction isotyping: the nucleotide substitution corresponding to position 192 (Glu-Arg) creates an AlwI restriction site (15). A 99 base-pair fragment covering the region containing the mutation was amplified by polymerase chain reaction (PCR): 34 cycles denaturation at 93°C for 1 min, annealing at 63°C for 30 sec, and extension at 72°C for 1 min. PCR products were digested with AlwI (New England Biolabs, Beverly, MA), separated by non-denaturing acrylamide gel (12%) electrophoresis, and visualized by ethidium bromide. Allele Q yielded a 99 bp fragment and allele R two 65 and 34 bp fragments.

Paraoxonase and arylesterase activities

Paraoxon hydrolysis (PON p.ase) and arylesterase (PON ar.ase) activities were measured according to Gan et al. (30), using, respectively, paraoxon or phenylacelate as substrates, in the presence of 1 mm Ca^{2+} in 100 mm Tris-HCl buffer (pH 8.0) for PON p.ase and 20 mm Tris-HCl buffer (pH 8.0) for PON ar.ase. Results are expressed as U/l for PON p.ase (nmol paraoxon hydrolyzed per min) and U/ml for PON ar.ase (μmol phenyl acetate hydrolyzed per min).

Preparation of lipoproteins

LDL (d 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation from freshly drawn normal plasma collected on EDTA. Isolated LDL were dialyzed against phosphate-buffered saline (PBS) with EDTA (0.1 mm) and stored in aliquots at −80°C. Prior to each experiment, EDTA was removed by overnight dialysis against excess EDTA-free PBS, pH 7.4, at 4°C under vacuum. The same LDL preparation presenting stable oxidation characteristics served in all experiments. HDL (d 1.063–1.21 g/ml) was isolated from the serum of individual NIDDM patients by sequential ultracentrifugation and dialyzed against PBS, pH 7.4, at 4°C.

Oxidation of LDL by Cu^{2+}

LDL (1.0 mg protein) was incubated with HDL (1.0 mg protein) from NIDDM patients for 6 h at 37°C in PBS containing 5.0 μmol/l $CuSO_4$ (final volume 1.0 ml). Blank consisted of LDL without copper or HDL, and control consisted of LDL incubated with copper alone for the same period (9).

Lipid peroxide concentrations were measured at the specified time points using the CHOD-iodide reagent method (31) on 0.1-ml samples of the reaction mixture. Oxidation was terminated by addition of 1.0 ml of reagent containing EDTA and butylated hydroxytoluene (24 μmol/L and 20 μmol/L, final concentrations). After 30 min incubation at room temperature in the dark, absorbance was measured at 365 nm and concentration of lipid peroxides was calculated using a molar extinction coefficient of 2.46×10^4. Protection was expressed as percent reduction in lipid peroxide production with respect to oxidation of LDL in the absence of HDL (control).

Anionic electrophoretic mobility of LDL was determined by electrophoresis on agarose gels and staining with Sudan Black (Beckman). The migration distance was measured by reference to origin, and changes in mobility were expressed relative to migration of native, non-incubated LDL.

Statistics

Differences in the time courses of LDL oxidation attributable to HDL and to PON1 genotype were analyzed by ANOVA for repeated measures. Differences at single time points were compared by paired *t*-test.

RESULTS

Paraoxonase and arylesterase activities in NIDDM patients

PON p.ase was 6-fold higher ($P < 0.0001$) in the serum of patients with the RR genotype (**Table 1**). PON ar.ase,

TABLE 1. Clinical characteristics of the patients

	QQ	RR	*P*
Age	58 ± 1	57 ± 1	NS
Sex (F/M)	3/9	6/6	
BMI (kg/m^2)	27.8 ± 1.8	27.9 ± 1.3	NS
HbA1c (%)	8.4 ± 0.6	8.1 ± 0.4	NS
Cholesterol (mmol/l)	5.3 ± 0.3	6.1 ± 0.3	NS
TG (mmol/l)	1.6 ± 0.2	2.2 ± 0.4	NS
LDL-C (mmol/l)	3.2 ± 0.2	3.7 ± 0.2	NS
HDL-C (mmol/l)	1.4 ± 0.2	1.3 ± 0.2	NS
PON p.ase (U/l)	47 ± 4.4	299.7 ± 23.7	<0.0001
PON ar.ase (U/ml)	77.7 ± 3.7	99.4 ± 5.4	0.015
PON p.ase/PON ar.ase	0.61 ± 0.05	3.04 ± 0.20	<0.0001

which is representative of enzyme–protein concentrations (15, 16) only displayed a 1.3-fold difference which was, nevertheless, significant ($P < 0.015$). Consequently, the ratio of PON p.ase to PON ar.ase, representative of the specific activity of PON, was 5-fold higher in patients with the RR genotype. After ultracentrifugal isolation of HDL, PON p.ase activity per g HDL protein was still 4-fold higher for the RR than QQ genotype (64.2 ± 2.4 vs. 15.1 ± 1.9 U/g protein, $P < 0.0001$), indicative of similar recoveries for both genotypes.

Effects of HDL from QQ and RR genotype patients on in vitro oxidation of LDL from type 2 diabetic patients

Cu^{2+} induced peroxidation of control LDL (protein 1 mg/ml) was reduced to the same extent by HDL (protein 1 mg/ml) from either genotype (n = 7 of each), whether evaluated by the accumulation of peroxides (**Fig. 1A**) or the increase in electrophoretic mobility of LDL particles (Fig. 1B) ($P < 0.0001$ for both, by ANOVA for repeated measurements). Detectable concentrations of peroxides only started appearing after 2 h and, in the presence of HDL, maximal protection was observed after 4 h of incubation, at which time accumulation of peroxides was reduced by 60.6 ± 20.0 and 63.9 ± 23.5%, respectively, in the QQ and RR groups (Fig. 1A). After 6 h of incubation respective protections only amounted to 27.6 ± 33.0 and 38.8 ± 38.6%. An identical trend was found in protection against increased mobility (**Fig. 2B**) which reached 61.3 ± 21.8 and 61.4 ± 26.5%, respectively, in the QQ and RR genotypic groups at 4 h and 45.7 ± 21.0 and 52.8 ± 31.0% at 6 h. PON p.ase was inactivated to the same extent in both groups during the course of incubation and only retained 31 and 32% of its activity at the end of the 6 h (not shown) while incubation of HDL alone with Cu^{2+} led to a similar loss of activity (not shown).

After 4 h of oxidative incubation, protection afforded by HDL (both genotypes considered together) was in direct correlation with PON p.ase in the medium: $r = 0.501$, $P = 0.011$ for inhibition of peroxide production and $r = 0.478$, and $P = 0.023$ for change mobility (Figs. 2A and B). Although the correlation existed in both genotypic groups taken separately, it only reached significance for the RR genotype, possibly due to the narrow range of activity in patients with the the QQ genotype. The slope of the correlation was, however, much steeper for the QQ-type enzyme, resulting in a same wide range of 20–90% protection in both groups. It would thus seem that if PON were to be related to protection of LDL against oxidation, the QQ-type enzyme should be more active than the RR-type.

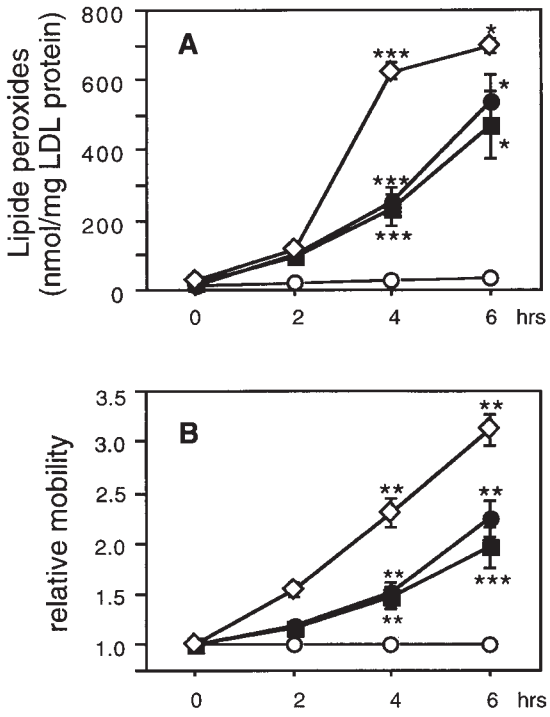

Fig. 1. Time course of LDL oxidation evaluted by (A) lipid peroxide accumulation in the medium; (B) change in electrophoretic mobility of LDL relative to native LDL. LDL (1.0 mg) from the same preparation was incubated for the indicated times with 5 μm Cu^{2+} in the presence or absence of HDL (1.0 mg) from type 2 diabetic patients with the QQ (n = 7) or RR (n = 7) genotype. (◇) LDL, no HDL; (●) LDL + QQ-HDL; (■) LDL + RR-HDL; (○) native LDL, no Cu^{2+}. ANOVA for incubation with or without HDL : $P < 0.0001$. Significant differences with incubation in the absence of HDL, at individual time-points by *t*-test: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

PON p.ase is thermolabile and Ca-dependent. In order to investigate whether protection was related to actual capacity to hydrolyze paraoxon, we studied the effect of PON p.ase inhibition in all samples. Incubation of HDL for 10 min at 56°C prior to the experiment resulted in almost total loss of PON p.ase (**Fig. 3A**). Protection of LDL against oxidation, however, remained unaffected (Fig. 3B). Further, this protection remained correlated with PON p.ase in the non-thermally treated sample ($r = 0.626$, $P < 0.05$ for peroxides; $r = 0.913$, $P < 0.0001$ for mobility). Identical results were obtained when HDL-PON p.ase was inactivated by EDTA removal of Ca followed by dialysis (not shown).

As HDL lost some of their protective power (Figs. 1A and B) during the course of the experiment as well as some of their PON p.ase activity (50% between 0 and 2 h and a further 10% between 4 and 6 h), we tested whether Cu^{2+} might not specifically alter the protective mechanism at the level of HDL. **Figure 4** shows that preincubation of HDL from a non-diabetic subject with QR genotype for either 2 or 4 h in the presence of copper before

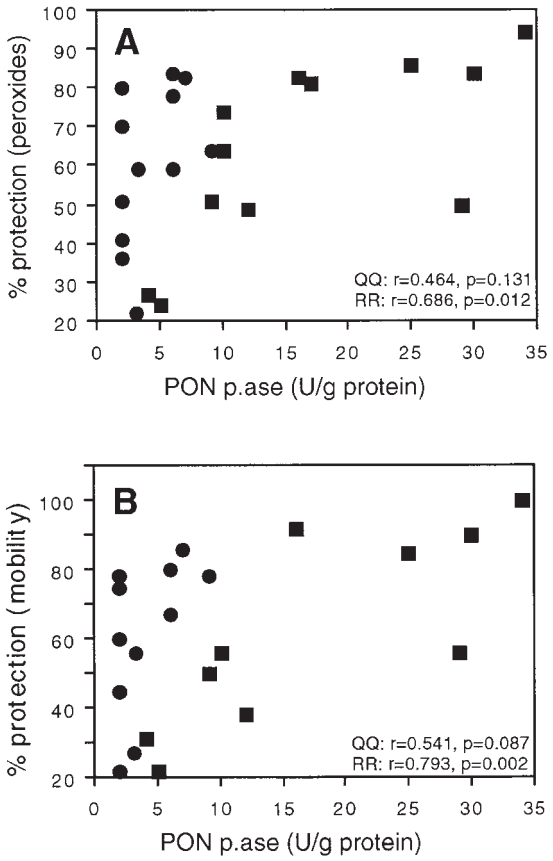

Fig. 2. Correlation between PON p.ase in the medium and protection afforded by HDL as evaluated by (A) production of peroxides; (B) change in electrophoretic mobility relative to native LDL. (●) QQ genotype; (■) RR genotype.

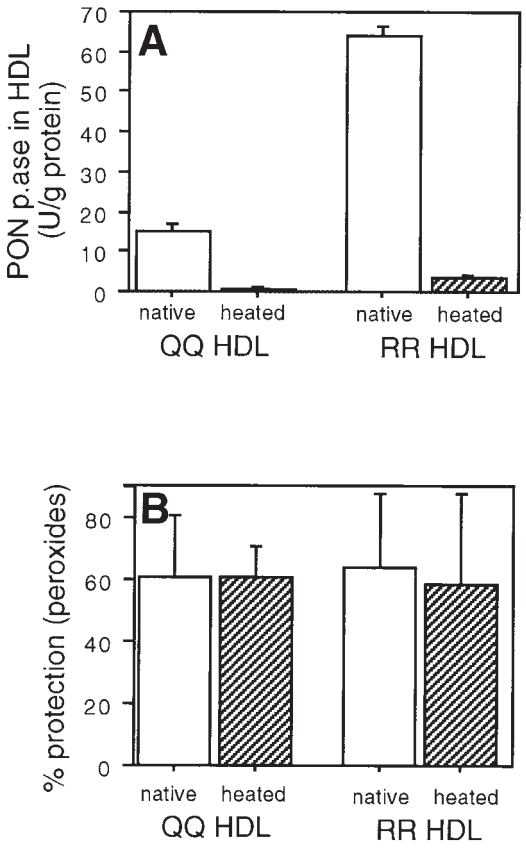

Fig. 3. Effect of thermal inactivation (56°C, 10 min) on (A) PON p.ase, (B) protection of LDL by HDL against Cu^{2+} oxidation.

addition of LDL left their antioxidant capacity unchanged, although PON p.ase was again reduced from 32 to 9 and 2 units, respectively, per g HDL protein after 2 and 4 h incubation with Cu^{2+}.

Dose–response of protection against oxidation by HDL

Insufficient amounts of HDL from patients being available to compare dose–responses between genotypes, this study was performed using concentrations of 0.5–3.0 mg of HDL-protein from normal subjects with QQ, QR, and RR genotypes during a 4 h incubation in the presence of LDL and 5 μm Cu^{2+}. In all cases, maximal protection of LDL was afforded by HDL concentrations of approximately 2 mg per ml (**Fig. 5A**). HDL from the QQ and QR subjects both provided 100% protection, but those from the RR subject provided at most only 70%. In light of the data in Fig. 2 showing similar ranges of protection by sera from QQ or RR patients, this should not, however, be taken as a specific property of the RR genotype. The same relationship was observed with PON ar.ase (not shown) which is representative of enzyme–protein concentrations. A striking difference was, however, detected when protection was expressed as a function of PON p.ase (Fig. 5B): maximal protection corresponded to far lower level of activity in the medium for the QQ genotype, intermediate for QR and high levels for RR.

Purified PON has been shown to protect HDL against oxidation and to inhibit LDL-induced monocyte migration in cocultures of vascular cells. The question remains whether PON is at all involved in the protection of LDL against oxidation by copper or whether the number of HDL particles or some other HDL component is responsible. This was addressed using HDL from rabbits that exhibit very high PON p.ase activity for small amounts of HDL protein. For this purpose, serum was obtained from a normal, chow-fed, New Zealand White rabbit and HDL were isolated according to the same procedure as in the case of human serum. Increasing amounts of HDL protein from 0.0625 to 1.0 mg were used in the incubation corresponding to PON p.ase ranging from 37.5 to 700 units. Maximal protection was obtained between 0.125 and 0.25 mg of HDL protein (Fig. 5A), for PON p.ase between 75 and 150 units (Fig. 5B), i.e., in the same range of activities as provides maximal protection with the human enzyme but with protein concentrations 16- to 8-fold lower. This strongly suggests that protection is closely associated with PON, more so than with HDL particles.

In summary, our results show *1*) that capacity of HDL to protect LDL from oxidation is independent of the 192 Q→R genotype of their PON1, *2*) that HDL remain protective even when their capacity for paraoxon hydrolysis is abolished, but *3*) that protection remains correlated with

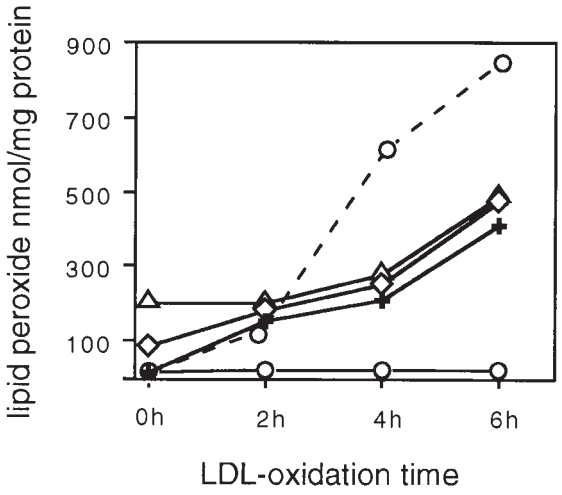

Fig. 4. Effect of preincubation of HDL with Cu^{2+} on their capacity to limit the production of peroxides by LDL. HDL (1.0 mg protein) were preoxidized with 5 μm Cu^{2+} for (+) 0, (◇) 2, or (△) 4 h before addition of LDL (1.0 mg protein). Controls (○) LDL without HDL, solid line: no Cu^{2+}, dashed line: 5 μm Cu^{2+}.

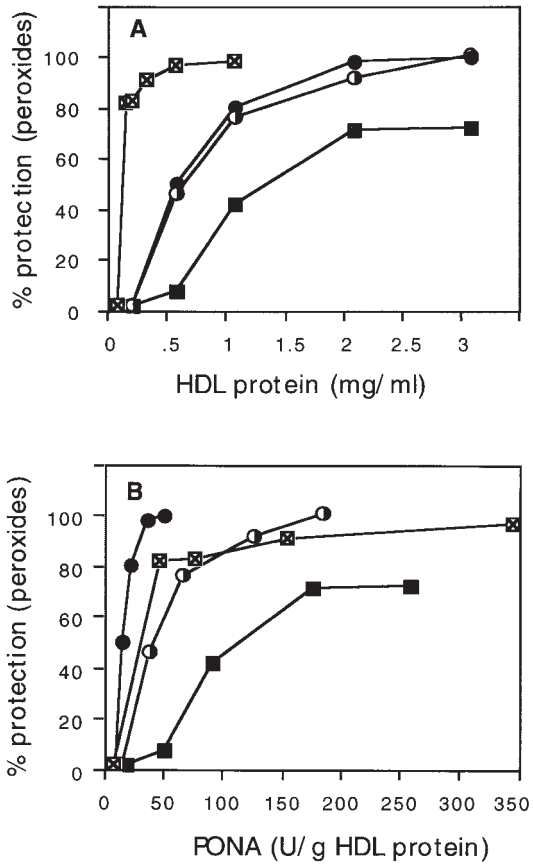

Fig. 5. Dose–response of LDL protection to increasing amounts of HDL-protein from non-diabetic subjects with (●) QQ, (◑) QR, or (■) RR genotypes and from (⊠) a rabbit. Percent protection with respect to oxidation of LDL in the absence of HDL, expressed relative to (A) HDL protein; (B) PON1 activity.

initial PON p.ase measured before inactivation and, finally, *4*) that rabbit HDL that have very high PON p.ase with respect to their protein content, are protective in proportion with PON p.ase and not with HDL protein.

DISCUSSION

PON1 genotypic variability specifies for either a glutamine (Q allele) or an arginine (R allele) in the 192 position. This alteration results in a low activity for the Q allele and a higher one for the R allele with no effect on arylesterase activity, implying different specific activities for the protein bearing PON p.ase of one or the other allotype. Although, within the QQ genotype, PON p.ase activity has been reported to vary as much as 13-fold in relation to actual protein concentration (15), there is no overlap between activities in the two homozygotic groups. If hydrolysis by PON of lipid peroxides is indeed responsible for the protective effect HDL exert towards LDL oxidation, then the prevalence of the high PON p.ase RR allele among diabetic subjects with CHD needs to be accounted for. It has been suggested that a more rapid loss of the protective quality by PON1 RR-HDL might offset the higher activity of the enzyme (30). Using LDL from a standardized source with a high degree of oxidability, we did not observe any difference between HDL from homozygotic subjects in the time course of enzyme inactivation or LDL protection.

Oxidized phospholipids and cholesteryl esters are presumably the main substrates for PON1 in its protective effect against oxidation of LDL (13, 27). When the effect of the two allozymes was tested on oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine, both were found to reduce to the same extent the capacity this oxidized phospholipid species has to induce monocyte migration in a coculture of human artery wall cells, although PON p.ase differed widely (29). This suggested that the protective effect of HDL on LDL oxidation was not related to the capacity for hydrolysis of paraoxon.

Our results reinforce this hypothesis. HDL from diabetic patients homozygote for both allozymes, whose mean PON p.ase differed by a factor of 6, displayed similar protective capacity towards peroxidation of LDL from a standardized source. Protection was verified when tested both by accumulation of peroxidative products in the incubation medium and by alteration of electrophoretic mobility of the LDL particles. Total inactivation of PON p.ase by Ca removal or mild thermal denaturation did not suppress the protective capacity of isolated HDL. Finally, we show that the loss of protection with incubation time is unrelated to copper inactivation of PON1 in HDL. Using transmigration of monocytes through co-cultures of arterial wall cells as a criterion for oxidative modification of LDL, Watson et al. (13) have shown that inhibition of PON p.ase in HDL to the extent of 90% by EDTA (or heating) only reduced their protective capacity from 73 to 46%. They further evidenced a cooperativity between PON and PAF acetyl hydrolase in the protective effect. On the basis of peroxide accumulation or particle charge, we did not

find inactivation of PON p.ase to reduce protection to any extent, rendering unlikely the contribution of PAF acetyl hydrolase to protection in this system. Recent results, however, show that specific non-competitive inhibitors of PON p.ase are able to totally suppress protection against oxidation both of LDL and HDL (27). Inhibition is therefore not equivalent to inactivation: whereas the latter appears to affect only the PON p.ase function, the former may also interfere with another function of PON, determinant for anti-peroxidative properties.

Incubation of HDL with copper prior to the introduction of LDL in the medium resulted in peroxidation of their lipids as well as inactivation of PON p.ase without any impairment of HDL protection. Such unimpaired protection by peroxidized HDL suggests that PON most probably does not act by hydrolyzing peroxides transferred to their own surface.

Although it does seem, in view of the most recent findings, that a function associated with PON plays a role in the antioxidant properties of HDL, the possibility remains that other HDL-associated proteins might play a determinant role. Indeed, we show that, using human HDL, maximal protection is afforded in all genotypic groups by equivalent amounts of HDL on the order of 2 mg, independent of PON p.ase activity. Apolipoprotein A-I reduces phospholipid peroxides in suspension to hydroxides (32) and both apo A-I and apoA-II reduce HDL peroxides to hydroxides by oxidizing methionines to their sulfoxides (33). Apolipoprotein J (apoJ) which is physically associated with PON in HDL (34) seems to prevent LDL-induced lipoperoxide formation in artery wall cells in coculture. It is greatly increased in cholesterol-fed rabbits or mice susceptible to atherosclerosis, while coronary patients display high ratios of plasma apoJ to PON (35). An interaction of apoJ with PON might contribute to modulation of its antioxidant activity, but evidence is at present too scarce to warrant speculation. Platelet activating factor protein (PAF-AH) may also play an enhancing role (13). Although many of the results obtained up to now, including some of ours, seem to weigh against a direct anti-oxidant effect of PON p.ase, there are arguments in favor of a close association. We do show that protection by HDL in which PON p.ase had been inactivated remained correlated with PON p.ase assayed in the non-heat-treated samples, and that in PON-rich rabbit serum, maximal protection is afforded by a minimal amount of HDL. Moreover, the dose–response experiments clearly show that, in the range of low HDL-protein concentrations, the antiperoxidative capacity is much higher, relative to PON p.ase for the QQ than RR genotype. In the physiological range of concentrations, however, a maximal effect is reached that is not different between genotypes. With a specific substrate to be defined, these observations could, no doubt, be translated in terms of kinetic parameters. The higher "apparent specific activity" of the antiperoxidative property in the QQ genotype might contribute to maintain a significant level of protection when HDL concentrations are markedly decreased. Such is the case in diabetic patients with confirmed coronary lesions and this might account for the apparently paradoxical prevalence of the R allele in this group.

Others have demonstrated a protective effect of semi-purified PON towards LDL-induced migration of monocytes in the vascular cell co-culture system (36), and, more recently, towards oxidation of HDL or of LDL (27). Moreover, inactivation of PON in HDL with a specific chemical inhibitor (27) deprived them of their protective quality against formation of dienes by LDL. Considered together, these results suggest that the protein bearing both PON p.ase, and PON ar.ase activities also possesses a third thermostable property, independent of arylesterase, closely associated with PON p.ase and unaffected by PON genetic variability. This last point is not unexpected as the mutation bears on a single codon located in the active part of the sequence and should mean that the active site responsible for protection is distinct from that responsible for paraoxon hydrolysis activity.

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