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Iron–Ascorbic Acid-Induced Oxidant Stress and Its Quenching by Paraoxonase 1 in HDL and the Liver: Comparison Between Humans and Rats

K. Trudel,[1] D. Sinnett,[2] R.W. James,[3] E. Delvin,[4] D. Amre,[2] E. Seidman,[2] and E. Levy[1]*

[1]Department of Nutrition, Université de Montréal, Montréal, Québec, Canada
[2]Department of Pediatrics, Université de Montréal, Montréal, Québec, Canada
[3]Lipid Laboratory of Clinical Diabetes Unit, University of Geneva, Geneva, Switzerland
[4]Department of Biochemistry, Université de Montréal, Montréal, Québec, Canada

Abstract Paraoxonase 1 (PON1) is a serum enzyme closely associated with high-density lipoprotein (HDL), which may protect against atherosclerosis by hydrolyzing lipid peroxides and several organophosphorus compounds. The purpose of the present study was to test the hypothesis that lipid peroxidation modifies the activity and protein mass of PON1 in humans and rats. Our findings revealed that the bulk of the activity monitored by the hydrolysis of paraoxon and phenyl acetate was confined to liver intracellular endoplasmic reticulum-derived microsomes and was mostly recovered in circulating HDL_3. Confirmation was obtained by the determination of PON1 expression by Western blot. It is noteworthy that PON1 levels were consistently decreased in human sera, HDL, and liver microsomes compared with rat counterparts. Concomitant with iron-ascorbate-mediated lipid peroxidation, there was a decline in PON1 activity and protein in both HDL_3 and microsomes, which was attenuated by butylated hydroxytoluene antioxidant treatment. The current data indicate that PON1 localization in microsomes and HDL_3 could represent a selective cellular and lipoprotein response to oxidative stress. This was tested by the iron-ascorbate oxygen-radical generating system. It is also proposed that the increased PON1 level may have a function related to the well-known atherosclerosis resistance of rats. J. Cell. Biochem. 96: 404–411, 2005.

Key words: PON1; oxidative stress; liver microsomes; HDL_3

Numerous studies emphasize the implication of oxidative stress in atherosclerosis and its resultant cardiovascular events [Stocker and Keaney, 2004]. Oxidative stress usually takes place when the production of harmful free radicals and additional oxidative molecules exceeds the capacity of antioxidant defenses. The effect of oxidative stress is removed by the antioxidant action of alimentary antioxidants as well as endogenous antioxidant enzymes [Djordjevic, 2004]. Exogenous antioxidants involve vitamin C, vitamin E, carotenoids, selenium, and others, whereas endogenous antioxidant defense mechanisms include enzymes such as catalase, glutathione peroxidase, and superoxide dismutase. Since observations emerging from several clinical studies stress the failure of antioxidant therapy in preventing cardiovascular morbidity and mortality [Blomhoff, 2005], the optimal approach at this time is to enhance endogenous antioxidants and reduce sources of oxidative stress.

Growing evidence indicates the critical role of high-density lipoprotein (HDL) in protection against atherosclerois and in the progression of coronary atherosclerotic disease [Hovingh et al., 2005]. If reverse cholesterol transport is believed to represent the crucial function of HDL, more and more studies underscore additional mechanisms through which HDL exerts its remarkable protective effect. This lipoprotein particle displays anti-inflammatory action

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*Correspondence to: Dr. E. Levy, GI-Nutrition Unit, CHU-Sainte-Justine, 3175 Côte Ste-Catherine, Montreal, Quebec, Canada H3T 1C5.
E-mail: emile.levy@recherche-ste-justine.qc.ca

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on the vasculature, inhibits thrombogenesis, and reduces the oxidative modification of low-density lipoproteins [Navab et al., 2001; Rohrer et al., 2004]. The antioxidative activity of HDL is intimately and primary linked to its component paraoxonase 1 (PON1).

PON1 is a calcium-dependent enzyme with a molecular mass of 43 KDa and its highest activity occurs in the liver and blood [Chemnitius et al., 1983]. It catalyzes the hydrolysis of organophosphates, aromatic carboxylic acid esters, and carbamates [La Du, 1992]. The precise process responsible for the cardioprotective action of PON is not clear, although it is likely to be related to its antioxidant strength. In fact, extensive in vitro data have demonstrated lower concentrations of lipid peroxides associated with LDL [Mackness et al., 1991] and HDL [Aviram et al., 1998], as well as a reduced pathobiologic influence of LDL [Watson et al., 1995] as a function of PON1 activity. More convincingly, animal models have revealed a greater degree of lipoprotein oxidation and more extensive atheroma formation in mice lacking PON1 activity [Shih et al., 1998], whereas overexpressing PON1 has a protective influence [Tward et al., 2002]. Only a few studies have analyzed the potential effect of lipid peroxidation on HDL-associated PON1. Similarly, little is known about the impact of lipid peroxidation on PON1 in the liver, the major source of enzyme synthesis. Furthermore, these investigations are limited to the analysis of activity measurements. Finally, the status of PON1 in the human liver and its response to oxidative stress have not received much attention. The purpose of the present study was to test the hypothesis that lipid peroxidation modifies the activity and protein mass of PON1 in HDL and hepatic microsomes that derive from humans and rats. To this end, HDL particles and microsomal fractions were isolated and incubated with iron/ascorbate, a widely used oxygen-radical generating system.

MATERIALS AND METHODS

Studies were carried out on human livers found invalid for transplantation and on animal livers obtained from Sprague–Dawley rats. The study protocol was approved by the ethics committees of Sainte-Justine Hospital and all experimental animal procedures were authorized by the Institutional Animal Care Committee.

Assay of Arylesterase Activity

Typically, 5 μl of serum were added to a total volume of 1 ml containing 10 mM phenyl acetate in 20 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$. In the case of ultracentrifugal fractions, the enzyme was assayed before dialysis using a 10–20 μl sample. An aliquot of each fraction was assayed (25–200 μl depending on the PON-1 activity). The increase in OD at 270 nm was monitored every 3 s for 30 s using a spectrophotometer. Activities are reported as units per liter, where 1 U is defined as 1 μmol of phenyl acetate hydrolyzed per minute.

Assay of Carboxylesterase Activity

Sera were diluted 160 times with 0.15 M NaCl, pH 7.4. A 20 μl aliquot was added to 200 μl of 0.48 mM *p*-nitrophenyl valerate in 50 mM HEPES, pH 7.0. The increase in OD at 405 nm was followed between 5 and 20 min. Activities are reported as units per milliliter, where 1 U is defined as 1 μmol of *p*-nitrophenyl valerate hydrolyzed per minute.

Preparation of Microsomes

Liver specimens were rinsed, homogenized, and centrifuged for 15 min at 12,000*g* at 4°C in order to prepare microsome fractions, a technique described earlier [Brunet et al., 1999, 2000]. The supernatant fraction was then centrifuged for 60 min at 100,000*g*. The pellet was centrifuged for 60 min at 4°C. The washed microsomal pellets were quick frozen and stored at 80°C for later use.

Lipoprotein Isolation

Lipoprotein fractions were isolated by discontinuous density gradient ultracentrifugation in an L5-65 preparative ultracentrifuge (Beckman, Montreal) with a Ti-50 rotor as reported previously [Levy et al., 1988, 1990]. HDL subpopulations were separated by centrifugation at 100,000*g* for 48 h at 4°C at the following densities: 1,125 g/L for HDL_2 and 1,210 g/L for HDL_3. The lipoprotein fractions were dialyzed intensively against 0.15 mol NaCl/L and 0.001 mol EDTA/L at pH 7.0.

Estimation of Lipid Peroxidation

The amount of free malondialdehyde (MDA) formed during the reaction was determined by HPLC, as we previously described [Bernotti et al., 2003]. Proteins were first precipitated

with a 10% sodium tungstate (Na_2WO_4) solution (Aldrich, Milwaukee, WI). The protein-free supernatants were then reacted with an equivalent volume of 0.5% (w/v) thiobarbituric acid solution (TBA; Sigma, St. Louis, MO) at 90°C for 60 min. After cooling to room temperature, the pink chromogene [(TBA) 2-MDA] was extracted with 1-butanol and dried over a stream of nitrogen at 37°C. The dry extract was then resuspended in a KH_2PO_4/methanol mobile phase (70:30, pH 7.0) before MDA detection by HPLC.

Statistical Analysis

All values were expressed as the mean ± SEM. The data were evaluated by ANOVA, where appropriate, and the differences between the means were assessed using the Student's two-tailed *t*-test.

RESULTS

Basal and salt-stimulated PON were measured in rats and humans (Fig. 1). We consistently observed that serum basal PON activity in rats is higher than in humans. Stimulation with calcium salt remained without effect on rat serum PON, while it doubled the basal human activity. The measurement of arylesterase also showed a superior activity level in rat sera.

A second series of experiments was undertaken to examine PON and arylesterase profiles in microsomal fractions (Fig. 2). Again, the enzymatic activities appeared significantly higher in rat microsomes than in human microsomes. The addition of calcium salt to the reaction mixture did not enhance PON magnitude in human and rat biological specimens.

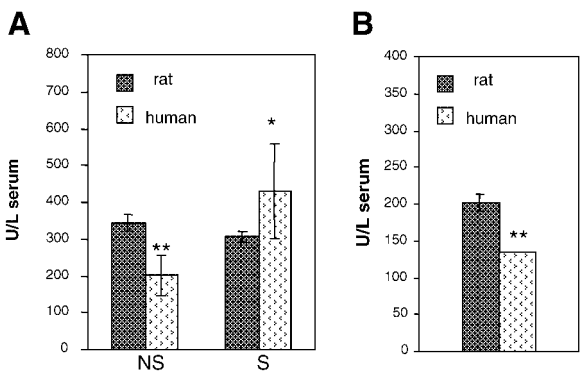

Fig. 1. Serum PON1 activity in humans and rats. The enzyme activity toward paraxon (**A**) and arylesterase (**B**) was determined by measuring the hydrolysis of diethyl-*p*-nitrophenyl phosphate and phenyl acetate, respectively. The activity toward paraxon was assessed in the presence (S) and absence (NS) of calcium salt. Values are expressed as mean ± SE for n = 7 in humans and n = 4 for rats. *$P < 0.05$ versus NS in humans; **$P < 0.01$ versus NS in rats.

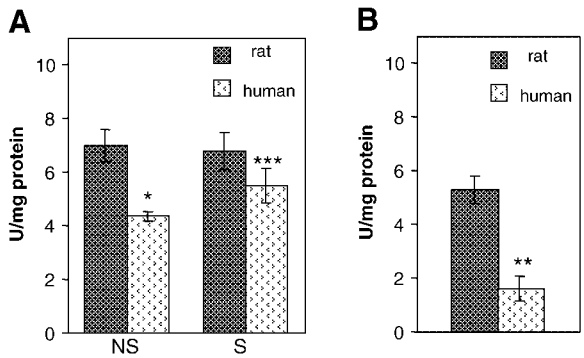

Fig. 2. Liver microsomal PON1 activity in humans and rats. Microsomes were prepared from liver homogenates and PON1 activity toward paraxon (**A**) and acylesterase (**B**) was determined by measuring the hydrolysis of diethyl-*p*-nitrophenyl and phenyl acetate, respectively. The activity toward paraxon was assessed in the presence (S) and absence (NS) of calcium salt. Values are expressed as mean ± SE for n = 4 in humans and n = 4 for rats. *$P < 0.05$ versus NS in rats; **$P < 0.01$ versus S in rats; ***$P < 0.05$ versus NS in humans.

Subsequent analyses were carried out to determine the PON1 protein concentrations. Specimens of sera, liver homogenates, and hepatic microsomes were electrophoresed on an SDS–polyacrylamide gel. An immunoblot of these samples showed immunoreactive bands corresponding to PON1 (Fig. 3). Densitometric estimation of the PON1 visualized on the immunoblot revealed that rat sera homogenates, and microsomes contained more PON1 protein levels than human counterparts. In fact, the Western blot results confirmed the enzymatic findings.

Since PON1 is located specifically on HDL particles in the circulation, we isolated HDL_2 (1,125 g/ml) and HDL_3 (1.210 g/ml) by ultracentrifugation and determined PON1 activity and protein mass. Both were associated with the denser subfraction of HDL in humans and rats, but far less in the former (Fig. 4).

It has been postulated that PON within HDL has a role in protecting LDL against oxidative modification. In the present study, we hypothesized that PON1 could guard blood intracellular organelles and HDL particles by scavenging peroxides caused by oxidative stress, which might affect its own concentration. In order to test this hypothesis, we exposed microsomes and HDL_3 to iron-ascorbate-mediated lipid peroxidation. Incubation with iron-ascorbate

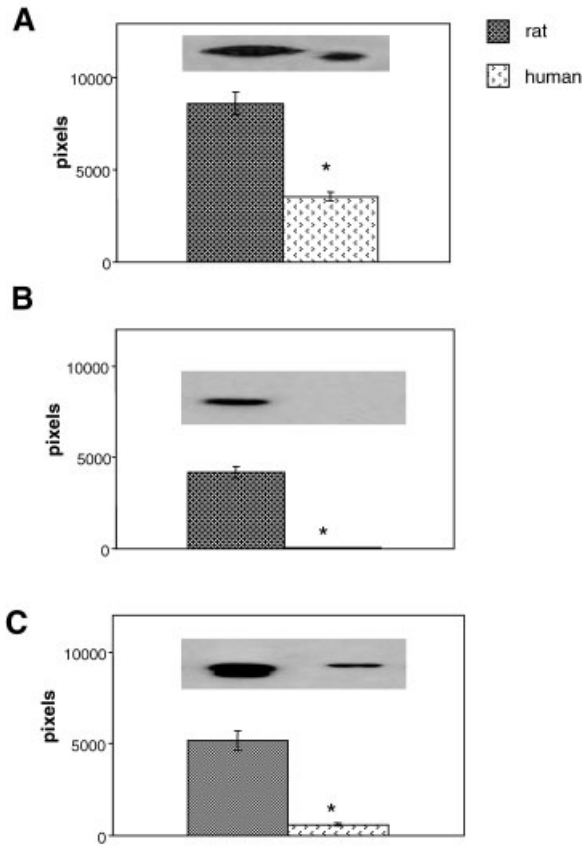

Fig. 3. Western blot analysis of human and rat PON1. To determine PON1 protein status, samples from sera (**A**), liver homogenates (**B**), and microsomes (**C**) were subjected to SDS–PAGE followed by Western blotting and then probed with antibodies. Immunoreactive proteins were made visible with horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence. PON1 mass was quantitated using an HP Scanjet scanner equipped with a transparency adapter and software. Values are expressed as mean ± SE for n = 4 in humans and n = 4 for rats. $^{**}P < 0.01$ versus rats.

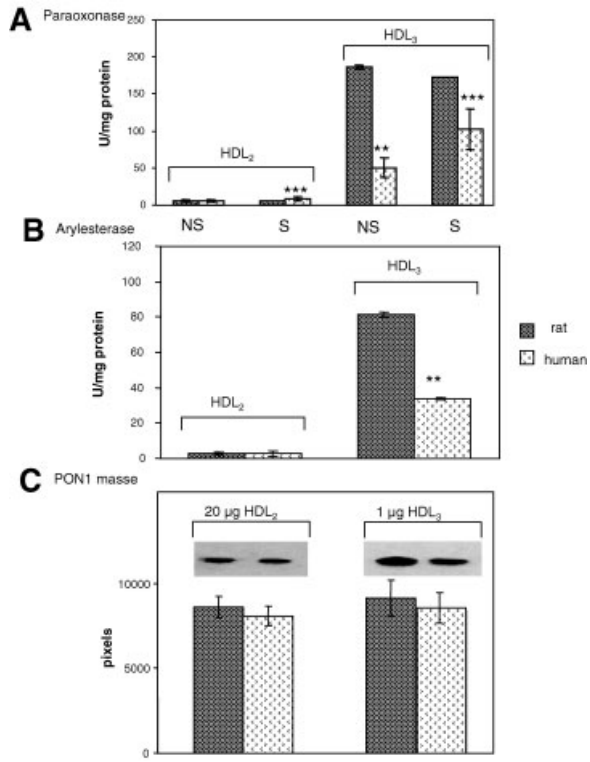

Fig. 4. PON1 activity and mass in HDL fractions. The enzyme activity toward paraxon (**A**) and arylesterase (**B**) was determined by measuring the hydrolysis of diethyl-*p*-nitrophenyl phosphate and phenyl acetate, respectively, in HDL_2 and HDL_3. The activity toward paraxon was assessed in the presence (S) and absence (NS) of calcium salt. PON1 mass (**C**) was quantitated following immunoblotting and scanning. Values are expressed as mean ± SE for n = 7 in humans and n = 4 for rats. $^{**}P < 0.01$ versus rat HDL_3; $^{***}P < 0.05$ versus (S) in rat.

at different concentrations (50–150 µM) resulted in a significant increase in malondialdehyde levels (results not shown) and inhibition of PON1 enzyme activities tested with paraxon and phenyl acetate (Fig. 5). PON1 downregulation was iron-ascorbate dose dependent. Although pre-incubation with butylated hydroxytoluene (BHT), a strong antioxidant, led to a protection against oxidative stress in humans and rats, it was unable to fully restore PON1 activity. The effects of iron-ascorbate treatment on PON1 protein expression were also examined in microsomes and HDL. The results shown in Figure 6 documented a decrease in PON1 expression response to oxidative stress-inducing agents. BHT normalized at least in part PON1 deterioration observed with iron-ascorbate treatment.

DISCUSSION

The liver is a key organ in lipid and lipoprotein metabolism. It is closely involved in the regulation of HDL synthesis and degradation. Recent reports have indicated that the enzyme PON1 is secreted in association with HDL and it represents a primary determinant of the antioxidant potential of this lipoprotein particle. In this study, we assessed the location of PON1 in the intracellular compartments of the hepatocyte, its distribution in HDL subpopulations and its response to oxidative stress. In particular, we focused on the human liver in comparison with the rat liver. Our findings showed that the bulk of the activity monitored by the hydrolysis of paraoxon and phenyl

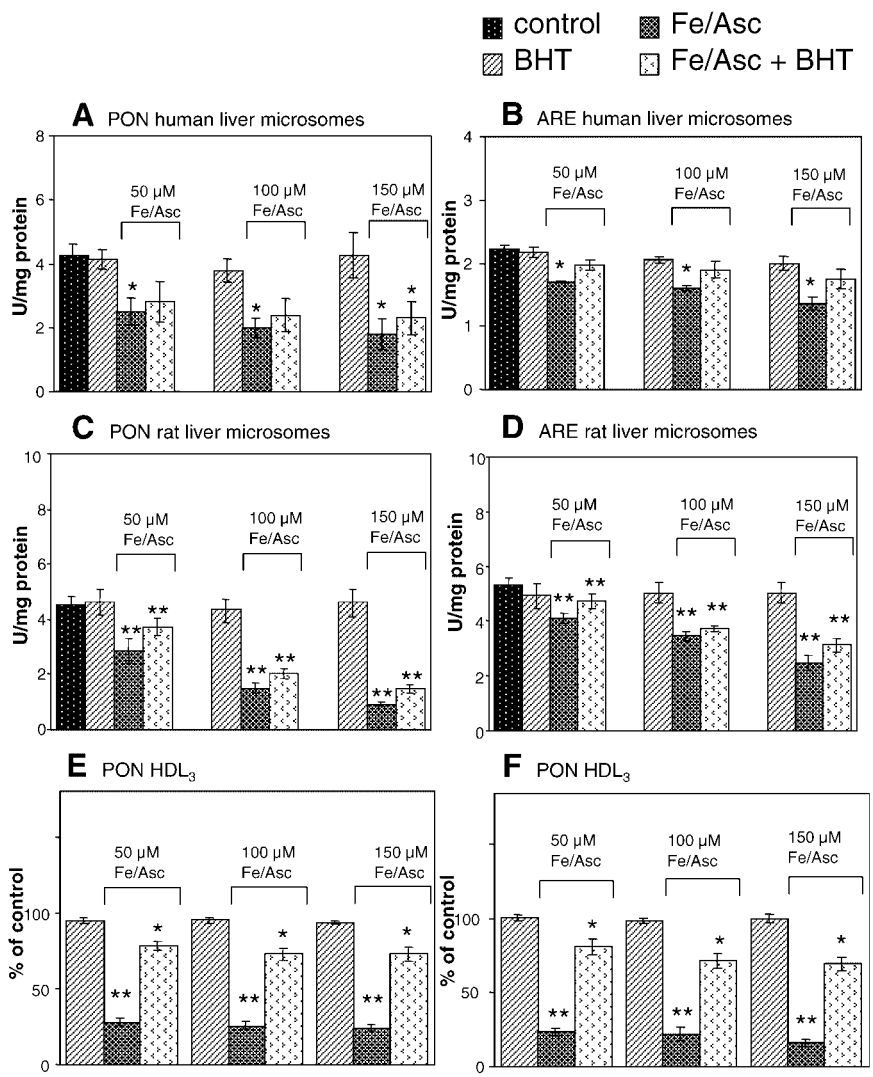

Fig. 5. Effect of iron-ascorbate on PON1 activity in liver microsomes and HDL_3 fractions. Microsomes (isolated from liver homogenates) and HDL_3 fractions (separated by ultracentrifugation) were challenged with various concentrations of iron-ascorbate. Then, the enzyme activity toward paraxon (PON) and arylesterase. Values are expressed as mean ± SE for n = 4–7 in humans and n = 4 for rats $^*P < 0.05$ and $^{**}P < 0.01$ when compared to controls.

acetate was recovered in circulating HDL_3 and confined to intracellular endoplasmic reticulum (ER)-derived microsomes. Confirmation was obtained by the determination of PON1 expression by Western blot. Our data also provided evidence that PON1 levels were consistently higher in rats than in humans. Finally, concomitant with iron-ascorbate-mediated lipid peroxidation, there was a decline in PON1 activity in HDL_3 and microsomes, which was partially corrected by BHT treatment.

According to our observations, PON1 is predominant in the microsomal fraction and HDL particles. Only negligible amounts were detected in other cellular compartments and lipoprotein classes such as LDL. It is, therefore, reasonable to assume that during HDL assembly in the ER compartment, there is a mechanism that regulates the addition of PON1 to the HDL particles. These data strengthen the suggestion that PON1 is mostly expressed in the liver and is carried in plasma bound to HDL [Reddy et al., 2001]. In this context, PON has been isolated from human plasma in association with apolipoprotein (apo) A-I and apo J [Blatter et al., 1993; Kelso et al., 1994]. Additional

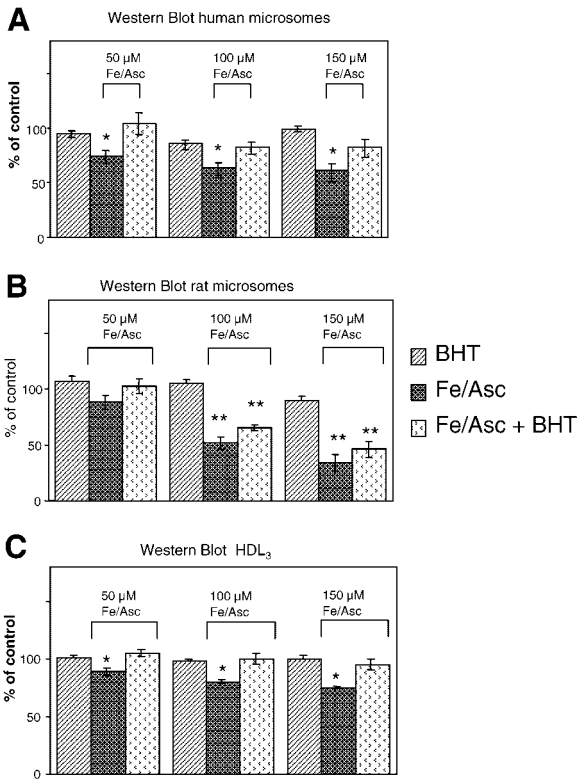

Fig. 6. Effect of iron-ascorbate on PON1 mass protein in human and rat liver microsomes and HDL_3 fractions. Microsomes and HDL_3 fractions were challenged with various concentrations of iron-ascorbate. Then, PON1 mass was quantitated following immunoblotting and scanning. Values are expressed as mean ± SE for n = 4 in humans and n = 4 for rats. $^*P < 0.05$ and $^{**}P < 0.01$ when compared to controls.

studies are necessary to delineate the mechanistic events involved in PON1 synthesis and incorporation in HDL.

Circulating HDL particles are heterogeneous in their physicochemical properties and antiatherogenic activities [Barter et al., 2003]. Indeed, small dense HDLs inhibit the expression of adhesion proteins by endothelial cells [Ashby et al., 1998] and potently protect atherogenic LDLs against oxidative stress [Kontush et al., 2003]. Consistent with our findings, the abundance of PON1 in HDL_3 may enhance its capacity to protect LDL from oxidation and preserve its important function. By exerting PON and arylesterase activities, PON1 tightly bound with HDL_3 can efficiently hydrolyze not only organophosphate compounds such as paraoxon and aromatic carboxylic acid esters, but also harmful peroxides.

The liver's unique metabolism has to tackle toxicity induced by drugs and xenobiotics [Gunawan and Kaplowitz, 2004]. In order to avoid an overwhelming lethal insult, the detoxification system made up of a large group of enzymes converts xenobiotics into metabolites and free radicals and participates in the excretion process. In coordination with detoxifying enzymes, antioxidants are involved in scavenging reactive species, thereby reducing cytotoxicity [Varga, 1992; Kehrer and Lund, 1994]. The presence of PON1 activity in liver microsomes may help hydrolyze a number of exogenous and endogenous chemicals and inactivate oxidative stress by-products.

Recently, we showed that iron/ascorbate-mediated lipid peroxidation altered the composition and properties of the bilayer lipid environment, affected the functions of sterol regulatory enzymes and integral membrane proteins of the ER and disturbed cholesterol homeostasis [Brunet et al., 1999, 2000]. Several laboratories have shown iron's ability to initiate strong lipid peroxidation, whereas ascorbic acid can amplify iron's oxidative potential by promoting metal ion-induced lipid peroxidation [Brasitus et al., 1985; Bachowski et al., 1988; Jourd'Heuil et al., 1993]. The data presented here clearly indicate that the iron-ascorbate system functioned not only as a producer of lipid peroxidation but, at the same time, diminished the activity and protein expression of PON1 in microsomes and HDL_3. The deterioration in PON1 resulting from the exposure of microsomes and HDL_3 to iron-ascorbate is probably attributable to oxidative stress, because the addition of the BHT antioxidant simultaneously prevented the occurrence of lipid peroxidation and improved the level of PON1. Iron-catalyzed lipid peroxidation may directly modulate enzyme activity by attacking polyunsaturated fatty acids, resulting in changes in the physical properties of the fluidity of the membrane in which PON1 is embedded [Poet et al., 2003]. Furthermore, iron-catalyzed lipid peroxidation may affect PON1 protein by disturbing its folding and accelerating its degradation.

In summary, our results show the profiles of PON1 in sera, HDL_3 and liver microsomes in humans and rats. A different behavior was noted between the two species as to PON1 levels, salt-stimulated serum PON1 activities and the inhibitory effect of lipid peroxidation. Not only did our data stress the localization of

PON1 particularly in human HDL_3 and liver ER, but they also emphasize that PON1 enzyme plays a significant role within the antioxidant systems in these specific sites.

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