

# Paraoxonase 1 (PON1) is a more potent antioxidant and stimulant of macrophage cholesterol efflux, when present in HDL than in lipoprotein-deficient serum: Relevance to diabetes

Mira Rosenblat, Rachel Karry, Michael Aviram\*

*The Lipid Research Laboratory, Technion Faculty of Medicine, The Rappaport Family Institute for Research in the Medical Sciences and Rambam Medical Center, 31096 Haifa, Israel*

Received 14 April 2005; received in revised form 12 July 2005; accepted 17 August 2005  
Available online 17 October 2005

## Abstract

The present study analyzed serum paraoxonase 1 (PON1) distribution among HDL and lipoprotein-deficient serum (LPDS) in atherosclerotic patients, and compared PON1 biological functions in these fractions.

Serum HDL and LPDS fractions were isolated from control healthy subjects, diabetic and hypercholesterolemic patients. PON1 activities and protein in HDL/LPDS, as well as its ability to protect against lipid peroxidation and to stimulate HDL/LPDS-mediated macrophage cholesterol efflux were measured. In LPDS from controls, PON1 protein and a significant paraoxonase activity were found, whereas arylesterase and lactonase activities were substantially reduced compared to HDL, by 78% and 88%, respectively. In diabetic patients, PON1 protein and paraoxonase activity in HDL were significantly decreased by 2.8- and 1.7-fold, respectively, compared with controls' HDL. In parallel, in these patient's LPDS, PON1 protein and paraoxonase activity were markedly increased by 3.7- and 1.7-fold, respectively, compared with controls' LPDS. PON1 in HDL (but not PON1 in LPDS) significantly decreased AAPH-induced lipid peroxides formation by 33%, and increased macrophage cholesterol efflux by 31%.

We conclude that PON1 is less antiatherogenic when present in LPDS than in HDL. The abnormal serum PON1 distribution in diabetic patients, could be responsible for the accelerated atherosclerosis development in these patients.

© 2005 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** Paraoxonase; HDL; LPDS; Lipids peroxides; Diabetes; Cholesterol efflux

## 1. Introduction

Paraoxonase 1 (PON1) is an HDL-associated esterase/lactonase [1–3] and its activity is inversely related to the risk of cardiovascular diseases [4,5]. Recently, PON1 was shown to be associated also with triglyceride-rich lipoproteins (chylomicrons and VLDL), but not with LDL [6,7]. The crystal structure elucidation of a variant of PON1 obtained by directed evolution, showed that PON1 consists of a six-bladed  $\beta$  propeller with a unique active site [8]. The role of PON1 in atherosclerosis development was demonstrated in studies, which used mice lacking PON1 [9,10], or over-

expressing PON1 [11,12]. PON1 antiatherogenic properties include protection of LDL, HDL and macrophages against oxidative stress [9,10,12–15], attenuation of oxidized-LDL uptake by macrophages [16], inhibition of macrophage cholesterol biosynthesis [17], and stimulation of HDL-mediated cholesterol efflux from macrophages [18]. Among HDL subfractions, HDL<sub>3</sub>, which is important in reverse cholesterol transport, carries the highest PON1 activity [19].

Most of serum PON1 is localized on the surface of HDL, and HDL major apolipoprotein, A-I (apoA-I) was shown to stabilize PON1 activity [1,2]. Under pathological conditions, such as in patients with low plasma apoA-I levels, PON1 distributed from small-size HDL to the LPDS [20]. In human apoA-I deficiency, 38% of PON1 protein was found in the

\* Corresponding author. Tel.: +972 4 8542970; fax: +972 4 8542130.  
E-mail address: aviram@tx.technion.ac.il (M. Aviram).

lipoprotein-free fraction, whereas in healthy subjects only 5% of total serum PON1 protein was in LPDS [21]. In humans and rabbits most of the PON1 arylesterase activity was shown to be HDL-associated, whereas in mice 30% of this activity was found in LPDS [22]. In the absence of apoA-I in mice, total PON1 arylesterase activity was reduced and over 60% was found in LPDS [22]. PON1 arylesterase activity and distribution were restored in apoA-I deficient mice following injection of adenoviruses encoding human apoA-I [22]. We hypothesized that PON1 distribution between HDL and LPDS could have important consequences on PON1 antiatherogenic properties. Thus, the aim of the present study was to analyze serum PON1 distribution between HDL and LPDS in atherosclerotic patients, and to assess PON1 functions when present in HDL versus in LPDS.

## 2. Methods

### 2.1. Subjects

Blood samples were collected from a control group consisted of three male healthy volunteers, three male NIDDM patients, and three male hypercholesterolemic patients. The controls were non-smokers, with no diabetes (serum glucose levels below 100 mg% and hemoglobin A1c levels were in the range of 4.8–6.2%), with no hypertension, or coronary artery disease, and they did not take any medications. The diabetes mellitus duration in the patients was 4–10 years with serum glucose levels above 160 mg% and hemoglobin A1c in the range of 7.5–11.3%. All patients had no ischemic heart disease, no hypercholesterolemia and were no smokers.

The hypercholesterolemic patients were no smokers, with no diabetes, hypertension, or coronary artery disease, and they did not take any medications. Their serum total and LDL cholesterol levels were above 270 mg/dl and 170 mg/dl, respectively, and their serum triglycerides levels were lower than 200 mg/dl. The serum samples were immediately analyzed for glucose, lipids (cholesterol and triglyceride) apolipoprotein A-I levels, PON1 activities and basal oxidative status. Then the serum samples were fractionated.

### 2.2. Isolation of serum lipoproteins

#### 2.2.1. Isolation of serum lipoproteins by fast-protein liquid chromatography (FPLC)

Serum lipoproteins were separated by size exclusion chromatography on an FPLC system [22]. Two hundred microliters of serum were diluted  $\times 2$  with phosphate buffered saline (PBS) and filtered. These diluted serum samples were fractionated through Superose 6 column (1 cm  $\times$  30 cm, Pharmacia) using prefiltered and degassed PBS pH 7.5. The flow rate was 0.5 ml/min, and sixty 0.5 ml fractions were collected and immediately analyzed for PON1 arylesterase and paraoxonase activities. VLDL was eluted between fractions

15–17, LDL between 23 and 26, HDL between 27 and 36, and LPDS between 37 and 60. The HDL peak is consisted of two peaks: the first one is HDL<sub>2</sub> and the second one HDL<sub>3</sub>.

#### 2.2.2. Isolation of serum lipoproteins by discontinuous density gradient ultracentrifugation (UC)

HDL and LPDS were prepared from human serum obtained from fasted normolipidemic volunteers, or diabetic patients by density gradient ultracentrifugation [23]. Solid KBr was added to 4 ml serum to increase its density to 1.25 g/ml. This solution was overlaid with 4 ml of  $d = 1.084$  g/ml KBr–NaCl solution, and overlaid with 4 ml of  $d = 1.006$  g/ml. The KBr solutions contained 2 mM of CaCl<sub>2</sub> and 100  $\mu$ M of diethylenetriaminepenta-acetic acid (DTPA) to preserve PON1 activity [24]. The tubes were centrifuged in a SW41 rotor (Beckman Coulter Canada Inc.) at 35,000 rpm (100,000  $\times g$ ) for 48 h at 4 °C. The VLDL, LDL, HDL and LPDS fractions were visualized, isolated and stored at 4 °C [23]. The HDL and LPDS fractions were dialyzed against 50 mM Tris–HCl, 2 mM CaCl<sub>2</sub>, pH 7.4, and their protein content was determined using the Folin phenol reagent [25].

### 2.3. PON1 preparation

#### 2.3.1. Human PON1

PON1 (a generous gift from Dr. Draganov, University of Michigan, Ann Arbor, USA) was purified from the sera of healthy human volunteers, by chromatography using Blue Agarose, DEAE and Con-A columns, as previously described [26].

#### 2.3.2. Evolved PON1

Evolved PON1 (a generous gift from Dr. Dan Tawfic from the department of Biological Chemistry, the Weitzman Institute of Science, Rehovot, Israel) was generated in *E. coli* after a directed evolution process as previously described [27].

#### 2.3.3. PON1 Western blot analysis

Western blot analysis was performed using SDS-PAGE, 10% bis-acrylamide gels. From the UC HDL or LPDS fractions, 20  $\mu$ l were loaded on the gel, and from the FPLC fractions 150  $\mu$ l were loaded. Blocking of the gel was in 2% BSA for 2 h at room temperature. The primary antibody (a generous gift from Dr. Draganov, Ann Arbor, Michigan, USA) was mouse monoclonal anti-human PON1 diluted (1:5000, v/v, in T-TBS with 1% BSA), and it was incubated with the membrane over night. The secondary antibody horseradish peroxidase-conjugated anti-mouse IgG (Sigma) diluted 1:5000 in T-TBS was incubated for 1 h at room temperature. The membranes were developed using the ECL Western blotting kit (Amersham). Two microliters (diluted 1:200) of purified hPON1 (3.8 mg/ml) were loaded as positive control. The membranes were exposed for 5 min.

#### 2.3.4. Arylesterase activity

Arylesterase activity was determined using 5  $\mu$ l serum or 10  $\mu$ l of the HDL or LPDS fraction in a total volume of 1 ml of 50 mM Tris–HCl, pH 8.0 containing 1 mM CaCl<sub>2</sub> and 1 mM phenyl acetate. The increase in OD at 270 nm was monitored along 1 min. One unit is defined as 1  $\mu$ mol of phenyl acetate hydrolyzed per milliliter per minute [26].

#### 2.3.5. Lactonase activity

Lactonase activity was determined using 10  $\mu$ l of serum or 50  $\mu$ l of HDL or LPDS fractions in a total volume of 1 ml of 50 mM Tris–HCl, pH 8.0 containing 1 mM CaCl<sub>2</sub> and 1 mM dihydrocoumarin. The increase in OD at 270 nm was then monitored along 1 min. One unit is defined as 1  $\mu$ mol of dihydrocoumarin hydrolyzed per milliliter per minute [3].

#### 2.3.6. Paraoxonase activity

Paraoxonase activity towards paraoxon was determined spectrophotometrically at 412 nm using 10  $\mu$ l serum or 50  $\mu$ l of HDL or LPDS fractions [26]. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute.

#### 2.4. Serum, HDL or LPDS lipids peroxidation

HDL or LPDS from healthy subject (5 paraoxonase U/ml) were incubated without or with 100 mM of 2,2'-azobis,2-amidinopropane hydrochloride (AAPH, Wako, Japan) for 2 h at 37 °C [28]. At the end of the incubation period, the amount of lipid peroxides [29] was measured. Basal serum lipid peroxidation status was measured by the thiobarbituric acid reactive substances (TBARS) assay [30].

#### 2.5. Cells

J774 A.1 murine macrophage cells were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% FCS and 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml, and 2 mM glutamine.

#### 2.6. Macrophage cholesterol efflux determination

J774 A.1 macrophages were incubated with [<sup>3</sup>H]-labeled cholesterol (2  $\mu$ Ci/ml) for 1 h at 37 °C, followed by cell wash in ice-cold PBS ( $\times$ 3) and a further incubation in the absence or presence of HDL or LPDS (5 paraoxonase U/ml) for 3 h at 37 °C [18]. Cellular and medium [<sup>3</sup>H]-labeled cholesterol was quantitated and the percentage of cholesterol efflux was calculated [the ratio of [<sup>3</sup>H]-label in the medium  $\times$  100 / ([<sup>3</sup>H]-label in the medium + [<sup>3</sup>H]-label in the cells)] [18]. HDL or LPDS-mediated cholesterol efflux represents the value obtained in the presence of HDL or LPDS minus the value obtained in cells incubated without HDL or LPDS.

#### 2.7. Statistics

Student's *t*-test was performed for all statistical analyses. Results are given as mean  $\pm$  S.D.

### 3. Experimental results

#### 3.1. Paraoxonase I (PON1) distribution in human serum from healthy subjects, diabetic patients and hypercholesterolemic patients

A representative fast protein liquid chromatography (FPLC) profile of serum lipoprotein fractions obtained from healthy subjects is shown (Fig. 1A). PON1 arylesterase and paraoxonase activities were measured in the lipoproteins and in the LPDS fractions (Fig. 1A and C). Whereas most of serum arylesterase activity was recovered in the HDL fraction, very low activity levels were found in the VLDL and LDL fractions, but up to 12% of total serum arylesterase activity was found in the LPDS fraction (Fig. 1B). Similar results were obtained for serum lactonase activity (data not shown). In contrast to the data obtained for serum distribution of arylesterase and lactonase activities, a substantial paraoxonase activity was noted in the LPDS fraction which could account for 44% of total serum paraoxonase activity (Fig. 1C). Paraoxonase activity in the VLDL and LDL fractions was very low (Fig. 1C).

HDL and LPDS were also isolated from the healthy subjects by discontinuous density-gradient ultracentrifugation (UC). Similar to the FPLC fractions results, also in the UC LPDS fraction, paraoxonase activity was almost the same as that obtained in HDL (Fig. 2A). In contrast, arylesterase activity was lower in LPDS than in HDL by 78% (Fig. 2B), and lactonase activity was lower in LPDS than in HDL by 88% (Fig. 2C). Upon comparing PON1 arylesterase activity in HDL obtained by FPLC versus UC (Figs. 1B and 2B), we found that per 1 ml serum the activity in the FPLC HDL was higher by 38% as compared to the activity observed in the UC HDL, showing loss of HDL-associated PON1 activity during the ultracentrifugation procedure.

The above results from FPLC and UC clearly indicate that PON1 in LPDS loses its arylesterase and lactonase activities and maintain its paraoxonase activity, whereas in HDL, PON1 possesses all three activities.

In order to prove that the LPDS paraoxonase activity is indeed related to PON1, we used the PON1 specific inhibitor 2-hydroxyquinoline (200  $\mu$ M). The PON1 inhibitor resulted in a significant 60% reduction in HDL arylesterase activity (from 22.4  $\pm$  1.5 to 9.0  $\pm$  1.0 U/ml), and also in a significant 76% reduction in LPDS arylesterase activity (from 2.9  $\pm$  0.5 to 0.7  $\pm$  0.7 U/ml). Similarly, paraoxonase activities in HDL and LPDS were also significantly reduced by the addition of PON1 inhibitor or mouse anti human PON1 monoclonal antibody (diluted 1:10, v/v) to these fractions (data not shown). These results clearly indicate that the

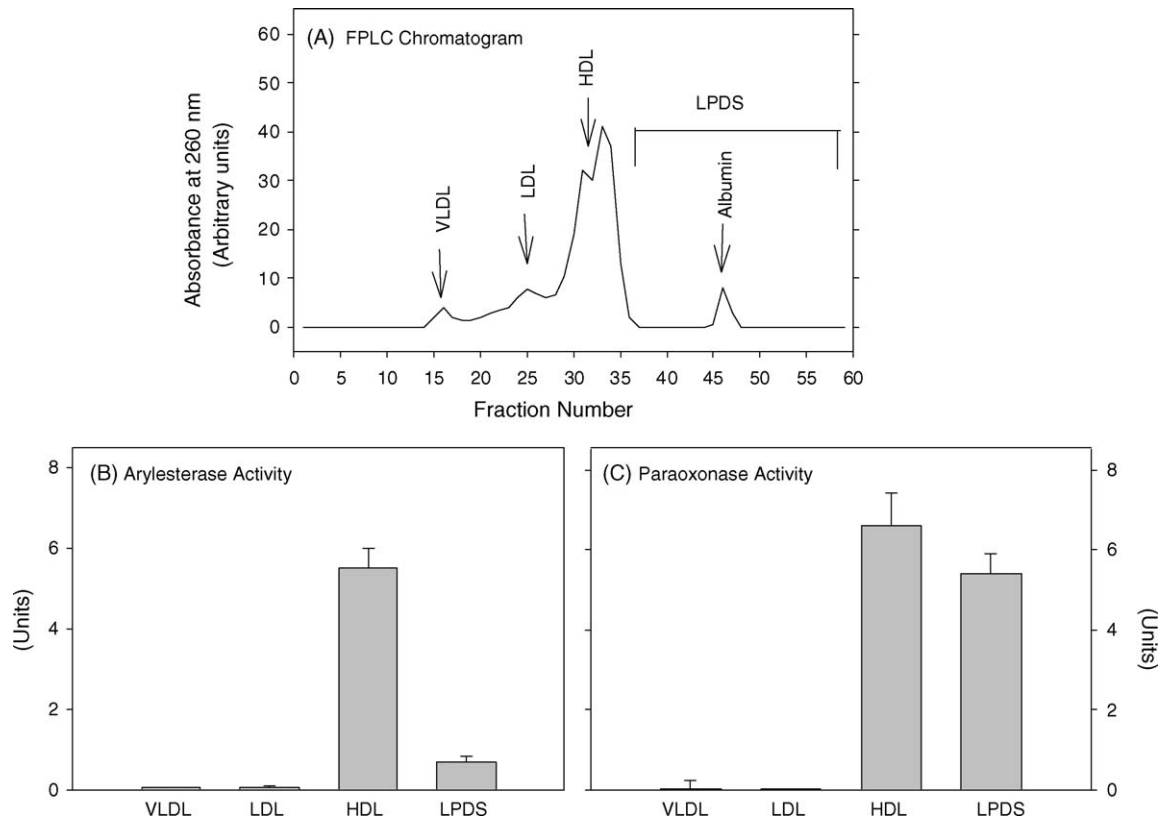


Fig. 1. Paraoxonase activities in lipoprotein fractions and in lipoprotein-deficient serum (LPDS) separated from the serum of healthy subjects by FPLC. Serum samples (100  $\mu$ l diluted  $\times 2$ ) from three healthy subjects were fractionated by FPLC and 60 fractions of 0.5 ml each were collected and the protein absorbance was monitored at 260 nm (A), arylesterase (B) and paraoxonase (C) activities were measured in all fractions. Results are given as mean  $\pm$  S.D. ( $n = 3$ ).

arylesterase/paraoxonase activity in LPDS, as well as in HDL is indeed related to PON1.

Under pathological conditions, the distribution of PON1 in serum between HDL and LPDS could have been altered, as was previously shown in patients with apoA-I deficiency [20,21]. Thus, we next analyzed PON1 distribution in serum from atherosclerotic patients, including diabetic and hypercholesterolemic patients. Increased oxidative stress was noted in the serum obtained from diabetic and hypercholesterolemic patients versus healthy controls, since the

basal serum TBARS levels in the diabetic and hypercholesterolemic patients were elevated by 116% and 72%, respectively, compared with the controls (Table 1). Serum paraoxonase, arylesterase and lactonase activities were significantly reduced in the diabetic patients by 46%, 51%, and 64%, respectively, compared with the activities measured in the controls (Table 1). In the hypercholesterolemic patients these activities were also reduced by 37%, 39% and 41%, respectively (Table 1). Serum apolipoprotein A-I (apoA-I) levels were significantly lower in the diabetic patients by 39%

Table 1

Serum oxidative stress, glucose, lipids and PON1 activities in diabetic patients and in hypercholesterolemic patients vs. healthy subjects

	Healthy subjects	Diabetic patients	Hypercholesterolemic patients
Oxidative stress (nmol TBARS/ml)	2.5 $\pm$ 0.2	5.4 $\pm$ 0.4*	4.3 $\pm$ 0.6*
Glucose (mg%)	83 $\pm$ 6	350 $\pm$ 20*	95 $\pm$ 7
Triglyceride (mg%)	113 $\pm$ 11	202 $\pm$ 5	178 $\pm$ 7
Total cholesterol (mg%)	191 $\pm$ 12	155 $\pm$ 27	268 $\pm$ 10*
LDL-cholesterol (mg%)	112 $\pm$ 21	88 $\pm$ 32	192 $\pm$ 5*
HDL-cholesterol (mg%)	50 $\pm$ 5	22 $\pm$ 3*	40 $\pm$ 3*
Apolipoprotein A-I (mg%)	170 $\pm$ 68	103 $\pm$ 10*	153 $\pm$ 20*
Paraoxonase activity (U/ml)	340 $\pm$ 37	183 $\pm$ 35*	208 $\pm$ 12*
Arylesterase activity (U/ml)	113 $\pm$ 16	55 $\pm$ 7*	71 $\pm$ 3*
Lactonase activity (U/ml)	22 $\pm$ 4	8 $\pm$ 3*	13 $\pm$ 3*

Serum samples were collected from three healthy subjects, three diabetic patients and three hypercholesterolemic patients. Basal serum oxidative status was determined by the TBARS assay. Results represent mean  $\pm$  S.D. ( $n = 3$ ).

\*  $p < 0.01$  vs. healthy subjects.

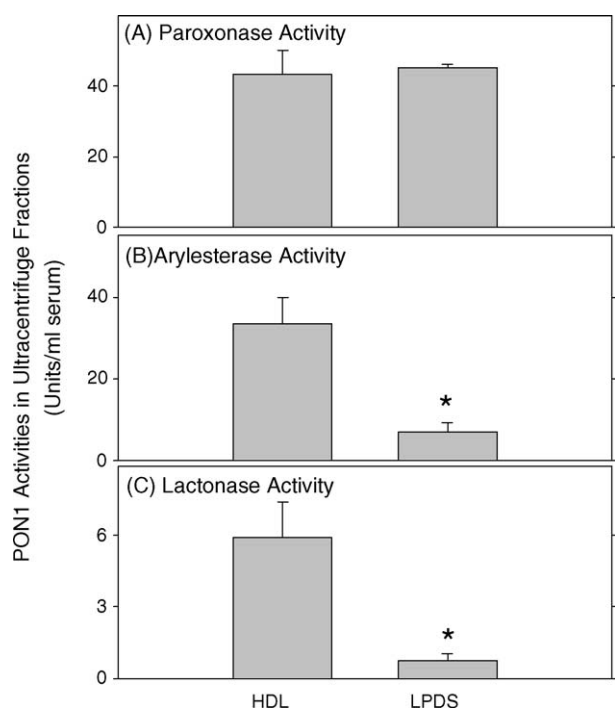


Fig. 2. Paraoxonase activities in the HDL and in the LPDS fractions separated from the serum of healthy subjects by density gradient ultracentrifugation. The serum samples (3.5 ml) of three healthy subjects were fractionated and paraoxonase (A), arylesterase (B), and lactonase (C) activities were measured in the HDL and LPDS fractions. Results represent the mean  $\pm$  S.D. ( $n=3$ ), and are given as units in fraction which originated from 1 ml of serum. \* $p < 0.01$  vs. HDL.

and in the hypercholesterolemic patients only by 10%, compared with the controls (Table 1). Serum HDL cholesterol levels were also significantly reduced in the diabetic and hypercholesterolemic patients by 56% and by 20%, respectively, compared with the levels present in healthy subjects (Table 1).

Using FPLC or ultracentrifugation fractionation, we next compared the distribution of PON1 between HDL and LPDS in serum from diabetic patients to that observed in healthy subjects (Fig. 3). In the FPLC system, paraoxonase activity in HDL from the diabetic patients (expressed as percentage of total activity in HDL + LPDS) was significantly reduced by 2.8-fold, compared to the control healthy HDL paraoxonase activity (Fig. 3A). In contrast, paraoxonase activity in the patients' LPDS fractions was significantly higher, by 1.7-fold, as compared to the control healthy LPDS (Fig. 3A). Similar results for paraoxonase activity were obtained upon serum fractionation by ultracentrifugation, with a 1.3-fold reduction in the diabetic patient HDL activity, and a 1.6-fold increase in the diabetic patient LPDS paraoxonase activity, compared with the activities observed in control healthy HDL or LPDS (Fig. 3B). We next measured apoA-I concentrations in the controls group and the diabetic patients HDL and LPDS samples. The apoA-I levels in the diabetic patient's HDL samples were reduced by 63%, compared with the apoA-I levels

in controls HDL ( $3.5 \pm 0.3$  versus  $9.5 \pm 2.4$  mg/fraction). In contrast, in the diabetic patient's LPDS apoA-I levels were increased by 21% compared with apoA-I levels in control LPDS ( $0.33 \pm 0.12$  versus  $0.27 \pm 0.06$  mg/fraction). In the diabetic patients, 8.4% of total serum apoA-I concentration were found in the LPDS fraction, in comparison to only 3.2% that were noted in control's LPDS, indicating increased dissociation of apoA-I (like PON1) from HDL to the LPDS fraction in diabetes versus controls. Paraoxonase activity in the diabetic patients HDL was reduced by 30%, in comparison to the activity observed in control's HDL when expressing it as units per mg of HDL apoA-I (data not shown). In hypercholesterolemic patients the distribution of PON1 in serum was found to be similar to that observed in the healthy controls subjects with 56% of total paraoxonase activity in HDL, and 44% in LPDS (data not shown).

In order to demonstrate that the paraoxonase activity in LPDS is indeed related to PON1 we performed Western blot analysis using PON1 monoclonal antibody. In the FPLC system, the amount of PON1 protein in the diabetic patient HDL was significantly reduced by 2.8-fold as compared with the control healthy HDL (Fig. 3C). In contrast, the amount of PON1 protein in the diabetic patient LPDS was increased by 3.7-fold as compared to the healthy control LPDS (Fig. 3C). Similar results were obtained for the ultracentrifugation fractions; PON1 protein was reduced by 2.5-fold in the diabetic HDL, or increased by 2.9-fold in the diabetic LPDS, compared with the control HDL or LPDS, respectively (Fig. 3D). These results correlate with PON1 paraoxonase activity in the HDL or LPDS fractions obtained by both serum fractionation methods. In the UC fractions, the PON1 protein content was considerably lower in LPDS versus HDL in both groups (controls and diabetic patients). However, the PON1 protein content in the control healthy LPDS was found to be about 5% of the total PON1 protein found in HDL + LPDS, whereas, in the diabetic patients, as much as 30% of the total PON1 protein in HDL + LPDS was found in their LPDS fractions (Fig. 3D).

The diabetic patient HDL (obtained by UC) contained  $8.6 \pm 2.0$  nmol lipid peroxides/ml compared with only  $0.8 \pm 0.1$  nmol/ml in the control healthy HDL ( $n=3$ ), suggesting that the increased oxidative stress in the diabetic patient HDL may be the result of its reduced PON1 levels and activity.

The increase in PON1 in LPDS (both protein and activity) in the diabetic patients versus healthy controls may be the result of a reduced capability of the diabetic HDL to bind PON1. To analyze this possibility, similar volumes of HDL from control or diabetic patient (separated by ultracentrifugation) were incubated with PON1 (50 paraoxonase U/ml) for 20 h at 37 °C, followed by a second ultracentrifugation in order to remove unbound PON1. Paraoxonase activity in the patient HDL (expressed as units per milligram of apoA-I) was increased by only 4% as compared to 127% increment in control HDL (data not shown), suggesting that the patient HDL is indeed less capable of binding PON1.

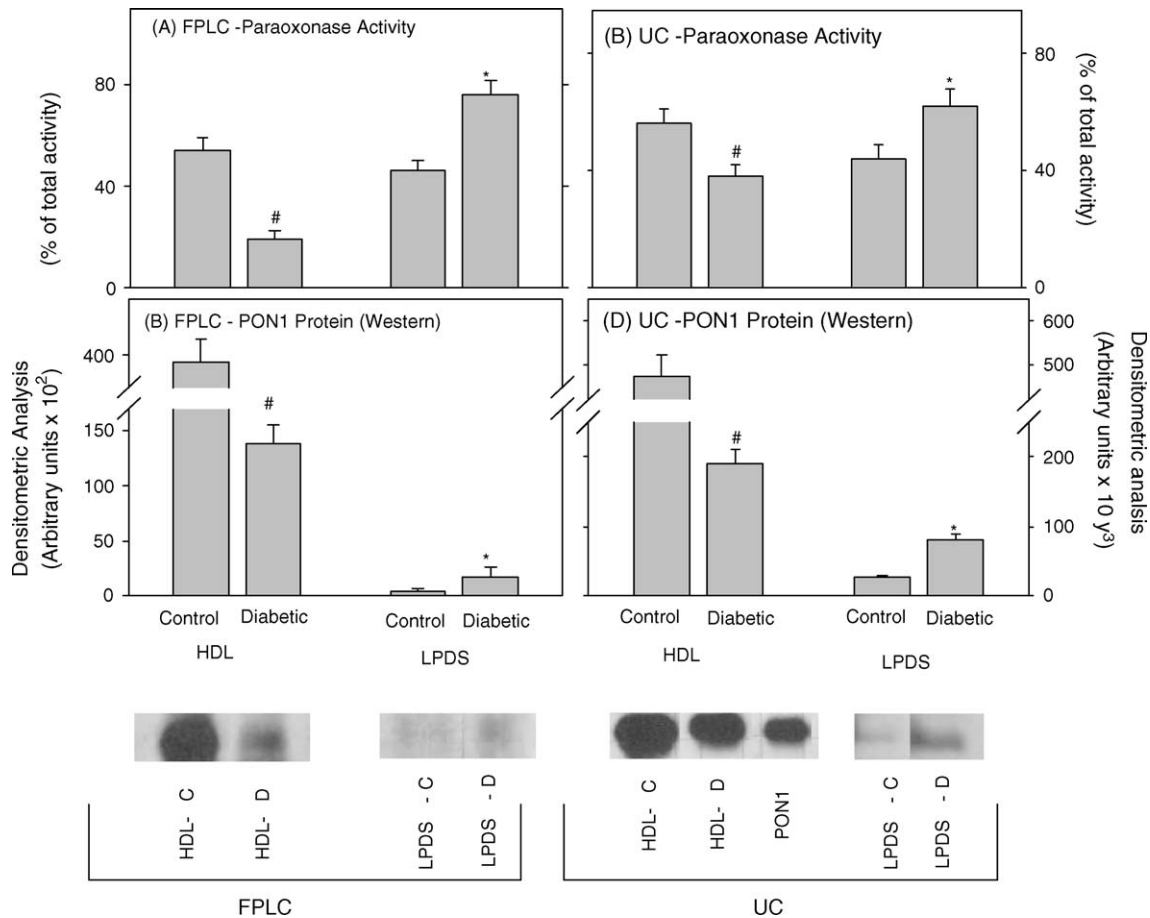


Fig. 3. Comparison between HDL and LPDS PON1 paraoxonase activity and protein in fractions isolated from the serum of healthy subjects and diabetic patients. The HDL and LPDS fractions were isolated from the serum of 3 healthy subjects (controls), or from three diabetic patients by FPLC or by density gradient ultracentrifugation (UC). Paraoxonase activity was determined in the FPLC HDL and LPDS fractions (A), as well as in the UC fractions (B). PON1 protein was analyzed by Western blot analysis, and the densitometric analysis of the protein bands, as well as the bands pictures are given for the FPLC (C) and UC (D) fractions. HDL-C: control HDL, HDL-D: diabetic HDL, LPDS-C: control LPDS, LPDS-D: diabetic LPDS. Results are presented as mean  $\pm$  S.D. <sup>#</sup> $p < 0.01$  diabetic HDL vs. control HDL. <sup>\*</sup> $p < 0.01$  diabetic LPDS vs. control LPDS.

### 3.2. PON1 in HDL but not in LPDS protects against lipid peroxidation

PON1 presence in HDL or in LPDS could have important consequences on PON1 antioxidant capability. Thus, we next analyzed the ability of PON1 in LPDS versus HDL (obtained from controls) to protect against AAPH-induced lipids peroxidation (Fig. 4A). HDL or LPDS (5 paraoxonase U/ml) were incubated without or with 50 paraoxonase U/ml of evolved PON1 for 2 h at 37 °C. Upon adding 100 mM of AAPH, the extent of lipid peroxidation in LPDS, measured by the lipid peroxides assay, was increased by 36% compared with HDL. In HDL incubated with the PON1, compared with control HDL (incubated without PON1,) a 33% decrement in AAPH-induced lipid peroxides level was noted (Fig. 4A). In contrast, enrichment of LPDS with PON1 had no significant effect on LPDS lipid peroxidation (Fig. 4A). Similar results were observed with purified human PON1 (data not shown). These results indicate that PON1 in HDL, but not lipoprotein-free PON1 in LPDS protects against lipid peroxidation.

To analyze whether HDL, in comparison to LPDS can stimulate PON1 paraoxonase activity, HDL or LPDS (5 paraoxonase U/ml) were incubated without or with PON1 for 2 h at 37 °C, followed by paraoxonase activity measurement (Fig. 4B). The observed paraoxonase activity of PON1 in HDL was significantly higher by 26%, compared to the calculated values (paraoxonase activities in HDL + that added as purified PON1, Fig. 4B). In contrast, the observed paraoxonase activity in LPDS was similar to the calculated value (Fig. 4B). These results suggest that HDL, but not LPDS, increases PON1 paraoxonase activity, probably by its ability to stabilize the enzyme.

### 3.3. PON1 in HDL stimulates cholesterol efflux from macrophages more than when present in LPDS

We have recently shown that HDL-associated PON1 has a stimulatory role in HDL-mediated cholesterol efflux from macrophages [18]. Thus, we next compared the ability of PON1 in LPDS versus PON1 in HDL (obtained from three

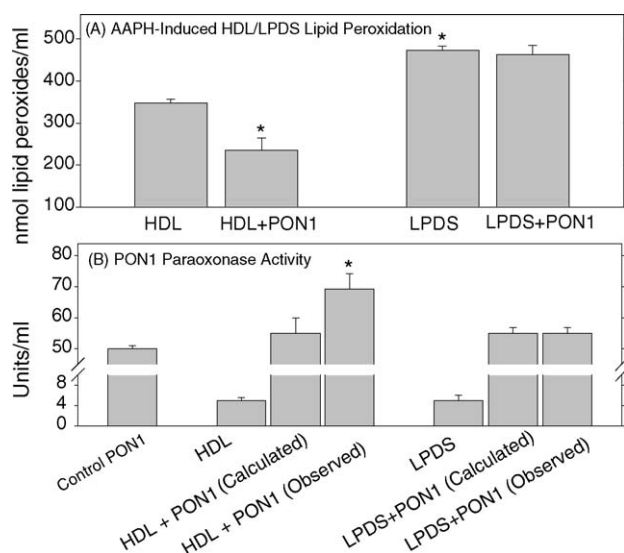


Fig. 4. The effect of HDL or LPDS enrichment with PON1 on their susceptibility to AAPH-induced lipids peroxidation. (A) HDL and LPDS (5 paraoxonase U/ml) were incubated with evolved PON1 (50 paraoxonase U/ml), as well as with the PON1 vehicle solution for 2 h at 37 °C. The HDL and LPDS samples were further incubated without or with 100 mM of AAPH for 2 h at 37 °C. The extent of AAPH-induced lipids peroxidation was measured by the lipid peroxides assay and calculated as described under the Methods section. (B) HDL and LPDS (5 paraoxonase U/ml) were incubated with evolved PON1 (50 paraoxonase U/ml) for 2 h at 37 °C, and paraoxonase activity was determined at the end of the incubation period. The calculated values are the sum of the values obtained for HDL or LPDS alone + added PON1 activity. Results are given as mean  $\pm$  S.D. of three different experiments. \* $p$  < 0.01 vs. HDL.

healthy subjects) to induce macrophage cholesterol efflux (Fig. 5). LPDS ability to induce cholesterol efflux from J774 A.1 macrophages compared to HDL (at a similar PON1 paraoxonase activity) was found to be 3.4-fold lower (Fig. 5). To analyze PON1 contribution to HDL/LPDS mediated macrophage cholesterol efflux, we have preincubated HDL or LPDS with PON1 inhibitor 2-hydroxyquinoline (200  $\mu$ M). Cholesterol efflux from the cells by HDL preincubated with the inhibitor was reduced by 25%, compared with the extent of cholesterol efflux by non-treated HDL (Fig. 5). In contrast, cholesterol efflux by LPDS preincubated with the PON1 inhibitor was reduced only by 10%, compared with the extent of cholesterol efflux by non-treated LPDS (Fig. 5). Furthermore, we next enriched the HDL or the LPDS fractions with similar paraoxonase activity of purified PON1. Cholesterol efflux from J774 A.1 macrophages by HDL enriched with PON1 (50 U/ml of paraoxonase activity) was 31% higher than that observed with HDL (that was not enriched with PON1, Fig. 5). In contrast, LPDS enrichment with a similar purified PON1 paraoxonase activity, increased cholesterol efflux from the cells by only 13%, compared with the value obtained on using LPDS that was not enriched with PON1 (Fig. 5). These results suggest that PON1 in HDL can stimulate macrophage cholesterol efflux significantly more than PON1 in LPDS.

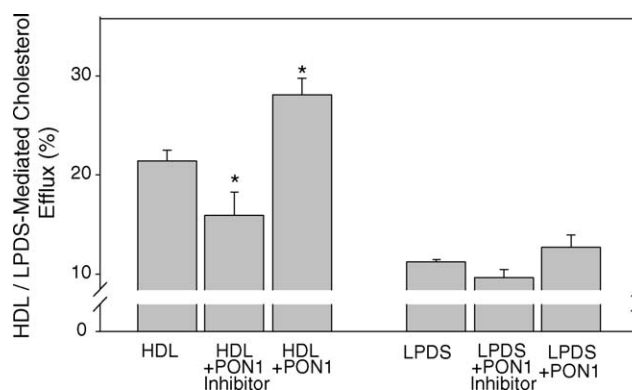


Fig. 5. Comparison between the ability of HDL and LPDS (derived from healthy subjects) to induce macrophage cholesterol efflux: effect of PON1 enrichment. HDL and LPDS were isolated from three healthy subjects by ultracentrifugation. J774 A.1 macrophages were incubated at 37 °C for 1 h with [<sup>3</sup>H]-cholesterol (2  $\mu$ Ci/ml) following by cell wash and a further incubation for 3 h at 37 °C without or with non-treated HDL fractions (100  $\mu$ g of protein/ml) or with non-treated LPDS fractions (at similar paraoxonase activity as in HDL). The cells were also incubated with HDL or LPDS fractions that were preincubated for 1 h at 37 °C with the PON1 inhibitor 2-hydroxyquinoline (200  $\mu$ M), or with HDL and LPDS that were enriched with evolved PON1 (50 paraoxonase U/ml). HDL or LPDS-mediated cholesterol efflux was then determined. Results are given as mean  $\pm$  S.D. of three different experiments. \* $p$  < 0.01 vs. HDL.

#### 4. Discussion

The present study demonstrated, for the first time, that in diabetes a significant amount of serum PON1 is dissociated from HDL to the LPDS fraction. Furthermore, we have shown that PON1 in LPDS, unlike PON1 in HDL, is not able to protect against lipids peroxidation, and to stimulate macrophage cholesterol efflux.

Western blot analysis in serum fractions revealed [6,7] PON1 presence mainly in HDL (>90%), but also in the triglyceride-rich lipoproteins chylomicrons (~1%) and in VLDL (~2%), and in the lipoprotein-deficient serum (LPDS, ~5%) [21]. The present study confirmed a similar content of PON1 protein in LPDS separated by ultracentrifugation, but lower levels (~1%) in LPDS prepared by FPLC. This phenomenon could be related to the ultracentrifugation forces that dissociate HDL surface constituents (apoA-I, phospholipids and also PON1) from the lipoprotein to LPDS. Furthermore, the activity of PON1 in the HDL obtained by ultracentrifugation was lower than in HDL from the FPLC and this could be related to loss of PON1 activity during the high-salt centrifugation. However, in both lipoprotein separation methods we observed the same phenomenon in the diabetic patients versus controls (Fig. 3).

An interesting finding of the present study is the significant paraoxonase activity observed in LPDS, up to similar levels as that observed in HDL. Paraoxon hydrolysis in LPDS is not specifically PON1 activity, and it may be related in part to a non-specific hydrolytic activity due to albumin. However, paraoxon hydrolysis is widely used to measure serum PON1

activity (although the serum contain albumin). Upon excluding the early phase of paraoxon hydrolysis we observed the same phenomena for the diabetic patients versus controls. Previous study [22] showed by using FPLC that there was almost no arylesterase activity in the LPDS fraction, and concluded that PON1 is completely associated with HDL. This study however did not measure paraoxonase activity which is considered to be a more specific activity of PON1 [31]. The present study also could not detect significant amount of arylesterase (as well as lactonase) activity in the LPDS fraction, but we measured substantial paraoxonase activity in this fraction, suggesting that PON1 conformational changes when present in LPDS versus HDL result in the loss of its arylesterase and lactonase activities, but stimulate its paraoxonase activity. It might be also that the lactonase/arylesterase activities of PON1 are more important than the paraoxonase activity to its physiological roles (in oxidation protection and cholesterol efflux), which are probably related to PON1-association with HDL [32].

Western blot analysis, as well as the use of PON1 specific inhibitor, or the mouse anti human PON1 antibody, all provided clear evidence that PON1 protein is indeed present not only in HDL, but also in LPDS, and the observed paraoxonase activity in LPDS, as in HDL is related to PON1.

HDL-associated PON1 was previously shown to protect against lipid peroxidation [9,10,12–15], a phenomenon that was attributed to its ability to hydrolyze specific oxidized lipids in lipoproteins [13,33], in macrophages [10,11] and in the atherosclerotic lesions [34]. We have also shown recently another antioatherogenic property of PON1, i.e. its ability to stimulate macrophage cholesterol efflux [18].

The present study demonstrated that PON1 in LPDS versus HDL loses its protection against LPDS or HDL lipid peroxidation, and its ability to stimulate macrophage cholesterol efflux. These phenomena could be related to the ability of HDL but not of LPDS, to stabilize PON1, secondary to the effect of apoA-I, the major apolipoprotein in HDL [1,2], as well as HDL-associated specific phospholipids, which bind the PON1 N-terminal leader sequence [1].

The lipids in LPDS that can be oxidized are various free phospholipids, or in association with unesterified cholesterol, as well as albumin-bound free fatty acids. The LPDS cholesterol acceptor which is responsible for macrophage cholesterol efflux could be free apoA-I as well as phospholipids.

Both diabetic and hypercholesterolemic patients are at high risk to develop accelerated atherosclerosis, which could be related to the increased oxidative stress observed in these patients [35–38]. However, although the extent of oxidative stress in diabetic and hypercholesterolemic patients was similar, the type of oxidative stress is different. This was evidenced by the type of reactive oxygen and nitrogen species (ROS/RNS) formed under these diseases, and the consequent different lipid peroxides formed. Such a different pattern of oxidative stress could be related to the abnormal distribution of PON1 between HDL and LPDS in diabetes, but not in hypercholesterolemia. Under both of the above diseases,

serum PON1 activity is decreased [35,36,38], a phenomenon which could be related to PON1 inactivation by oxidized lipids [39].

In diabetic patients, serum paraoxonase, arylesterase and lactonase activities, were all significantly lower than the activities measured in control healthy subjects, and also lower than those activities in hypercholesterolemic patients. Furthermore, apoA-I concentration in the diabetic patients was substantially reduced in comparison to controls and hypercholesterolemic patients. ApoA-I deficiencies in human and mice, were associated with a significant reduction in serum PON1 activities [1,20–22]. Furthermore, as shown in the present study in diabetes, not only serum PON1 activities were reduced in comparison to control healthy subjects, but also the distribution of PON1 from HDL to LPDS was clearly demonstrated, as was shown previously in human and mice apoA-I deficiencies [20–22]. The dissociation of PON1 from the diabetic HDL to LPDS could be the result of reduced apoA-I levels in HDL, and/or of HDL lipid peroxidation. Furthermore, glycation of HDL or directly of PON1 in HDL as occur in diabetes may result in detachment of PON1 itself from the HDL and PON1 inactivation [40]. We have shown that the diabetic HDL was less able to bind PON1, even when expressing the activity per mg of HDL apoA-I, further indicating that the diabetic HDL (unlike normal HDL) has a poor capability of stabilizing PON1 activity.

The PON1 activity in the diabetic patients HDL was significantly reduced, compared with control's HDL even when expressing it per HDL apoA-I content, suggesting PON1 dissociation in diabetic patients from HDL to the LPDS. However, the enzyme never dissociates itself from the lipid environment, and indeed it was shown to be associated with phospholipids (Getz and Reardon [4]). In the diabetic patients' LPDS we observed increased amount of apoA-I, compared with the controls' LPDS, which was paralleled by increased amount of PON1. Thus, we suggest that the PON1 in LPDS is associated with apoA-I and phospholipids. Furthermore, LPDS PON1 can be also associated with very high-density lipoproteins which are heavier and much smaller particles in size than HDL<sub>2</sub> and HDL<sub>3</sub>.

We have previously shown that PON1 protects against atherosclerosis, by its ability to reduce macrophage foam cell formation (via reducing oxidative stress and stimulation of cholesterol efflux from macrophages; [10,12,15,18]). The present study observations, suggest that PON1 association with HDL is important for PON1 ability to perform its anti-atherogenic effects, and dissociation of PON1 from HDL to the LPDS fraction is accompanied by the loss of PON1 anti-atherogenic properties.

Further studies thus should question whether appropriate means to increase HDL apoA-I and to reduced specific types of oxidative stress (such as that present in diabetes) can change the distribution of PON1 in serum towards HDL-association, a pattern which is related to enhanced PON1 anti-atherogenicity.

## Acknowledgments

We thank Dr. Aviv Shaish and Dr. Dror Haratz (from the Lipids Institute at Sheba Hospital, Tel Hashomer, Israel) for their assistance with the FPLC analysis.

## References

- [1] Sorenson RC, Bisgaier CL, Aviram M, et al. Human serum paraoxonase/arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids: apolipoprotein A-I stabilizes activity. *Arterioscler Thromb Vasc Biol* 1999;19:2214–25.
- [2] James RW, Deakin SP. The importance of high-density lipoprotein for paraoxonase-1 secretion, stability, and activity. *Free Radic Biol Med* 2004;37:1986–94.
- [3] Teiber JF, Draganov DI, La Du BN. Lactonase and lactonizing activities of human serum paraoxonase 1 (PON1) and rabbit serum PON3. *Biochem Pharmacol* 2003;66:887–96.
- [4] Getz GS, Reardon CA. Paraoxonase, a cardioprotective enzyme: continuing issues. *Curr Opin Lipidol* 2004;15:261–7.
- [5] Mackness M, Durrington PN, Mackness B. Paraoxonase 1 activity, concentration and genotype in cardiovascular diseases. *Curr Opin Lipidol* 2004;15:399–404.
- [6] Fuhrman B, Volkova N, Aviram M. Paraoxonase 1 (PON1) is present in postprandial chylomicrons. *Atherosclerosis* 2005;180:55–61.
- [7] Deakin S, Moren X, James RW. Very low density lipoproteins provide a vector for secretion of paraoxonase-1 from cells. *Atherosclerosis* 2005;179:17–25.
- [8] Harel M, Aharoni A, Gaidukov L, et al. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat Struct Mol Biol* 2004;11:412–9.
- [9] Shih DM, Xia YR, Miller E, et al. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem* 2000;275:17527–35.
- [10] Rozenberg O, Rosenblat M, Coelman R, et al. Paraoxonase (PON1) deficiency is associated with increased macrophage oxidative stress: studies in PON1-knockout mice. *Free Radic Biol Med* 2003;34:774–84.
- [11] Tward A, Xia YR, Wang XP, et al. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation* 2002;106:484–90.
- [12] Rozenberg O, Shih SD, Aviram M. Paraoxonase 1 (PON1) attenuates macrophage oxidative status: studies in PON1 transfected cells and in PON1 transgenic mice. *Atherosclerosis* 2005;181:9–18.
- [13] Aviram M, Rosenblat M, Bisgaier CL, et al. Paraoxonase inhibits high density lipoprotein (HDL) oxidation and preserves its functions: a possible peroxidative role for paraoxonase. *J Clin Invest* 1998;101:1581–90.
- [14] Mackness MI, Arrol S, Abbott CA, Durrington PN. Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis* 1993;104:129–35.
- [15] Aviram M, Rosenblat M. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. *Free Radic Biol Med* 2004;37:1304–16.
- [16] Fuhrman B, Volkova N, Aviram M. Oxidative stress increases the expression of the CD36 scavenger receptor and the cellular uptake of oxidized LDL in macrophages from atherosclerotic mice: protective role of antioxidants and of paraoxonase. *Atherosclerosis* 2002;161:307–16.
- [17] Rozenberg O, Shih DM, Aviram M. Human serum paraoxonase (PON1) decreases macrophage cholesterol biosynthesis: a possible role for its phospholipase-A<sub>2</sub> activity and lysophosphatidylcholine formation. *Arterioscler Thromb Vasc Biol* 2003;23:461–7.
- [18] Rosenblat M, Vaya J, Shih DM, Aviram M. Paraoxonase 1 (PON1) enhances HDL-mediated macrophage cholesterol efflux via the ABCA1 transporter in association with increased HDL binding to the cells: a possible role for lysophosphatidylcholine. *Atherosclerosis* 2005;179:69–77.
- [19] Bergmeier C, Siekmeier R, Gross W. Distribution spectrum of paraoxonase activity in HDL fractions. *Clin Chem* 2004;50:1–7.
- [20] James RW, Blatter Garin MC, Calabresi L, et al. Modulated serum activities and concentrations of paraoxonase in high density lipoprotein deficiency states. *Atherosclerosis* 1998;139:77–82.
- [21] Noto H, Hashimoto Y, Satoh H, et al. Exclusive association of paraoxonase 1 with high-density lipoprotein particles in apolipoprotein A-I deficiency. *Biochem Biophys Res Commun* 2001;289:395–401.
- [22] Cabana VG, Reardon CA, Feng N, et al. Serum paraoxonase: effect of the apolipoprotein composition of HDL and the acute phase response. *J Lipid Res* 2003;44:780–92.
- [23] Aviram M. Plasma lipoprotein separation by discontinuous density gradient ultracentrifugation in hyperlipoproteinemic patients. *Biochem Med* 1983;30:111–8.
- [24] Connelly PW, Maguire GF, Draganov DI. Separation and quantitative recovery of mouse serum arylesterase and carboxyesterase activity. *J Lipid Res* 2004;45:561–6.
- [25] Lowry OH, Rosenberg NJ, Farr L, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:225–65.
- [26] Gan K, Smolen A, Eckerson HW, La Du BN. Purification of human serum paraoxonase/arylesterase: evidence for one esterase catalyzing both activities. *Drug Metab Dispos* 1991;19:100–6.
- [27] Aharoni A, Gaidukov L, Tokar L, et al. Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proc Natl Acad Sci* 2003;101:482–7.
- [28] Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci USA* 1988;85:9748–52.
- [29] El-Saadani M, Esterbauer N, El-sayed M, et al. Spectrophotometric assay for lipid peroxides in serum lipoproteins using commercially available reagent. *J Lipid Res* 1989;30:627–30.
- [30] Buege JA, Aust AD. Microsomal lipid peroxidation. *Methods Enzymol* 1978;52:302–10.
- [31] Draganov DI, Teiber JF, Speelman A, et al. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J Lipid Res* 2005;46:1239–74.
- [32] Aviram M, Rosenblat M. Paraoxonases (PONs) and cardiovascular diseases: pharmacological and nutritional influences. *Curr Opin Lipidol* 2005;16:393–9.
- [33] Ahmed Z, Ravandi A, Maguire GF, et al. Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (PON1) during high density lipoprotein oxidation with peroxyxynitrite donor. *J Biol Chem* 2001;276:24473–81.
- [34] Aviram M, Hardak E, Vaya J, et al. Human serum paraoxonase (PON1), Q and R selectively decrease lipid peroxides in coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation* 2000;101:2510–7.
- [35] Letellier C, Duron MR, Jouanolle AM, et al. Serum paraoxonase activity and paraoxonase gene polymorphism in type 2 diabetic patients with or without vascular complications. *Diabetes Metab* 2002;28:297–304.
- [36] Tsuzura S, Ikeda Y, Suchiro T, et al. Correlation of plasma oxidized low-density lipoprotein levels to vascular complications and human serum paraoxonase in patients with type 2 diabetes. *Metabolism* 2004;53:297–302.
- [37] Ferretti G, Bacchetti T, Busni D, et al. Protective effect of paraoxonase activity in high-density lipoproteins against erythrocyte membranes peroxidation: a comparison between healthy subjects

- and type 1 diabetic patients. *J Clin Endocrinol Metab* 2004;89:2957–62.
- [38] Rosenblat M, Hayek T, Hussein K, Aviram M. Decreased macrophage paraoxonase 2 expression in patients with hypercholesterolemia is the result of their increased cellular cholesterol content: effect of atorvastatin therapy. *Arterioscler Thromb Vasc Biol* 2004;24:175–80.
- [39] Aviram M, Rosenblat M, Billecke S, et al. Human serum paraoxonase (PON1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radic Biol Med* 1999;26:892–904.
- [40] Ferretti G, Bacchetti T, Marchionni C, et al. Effect of glycation of high density lipoproteins on their physicochemical properties and on paraoxonase activity. *Acta Diabetol* 2001;38:163–9.