

Applied nutritional investigation

Pomegranate juice polyphenols increase recombinant paraoxonase-1 binding to high-density lipoprotein: Studies in vitro and in diabetic patients

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Abstract

Objective: The high-density lipoprotein (HDL)-associated paraoxonase-1 (PON1)/free PON1 ratio is lower in diabetic patients in comparison with healthy controls. Because diabetes is associated with increased oxidative stress, we hypothesized that a labeled recombinant PON1 (rePON1) would detect differences in HDL capacity to bind PON1 under specific experimental conditions, such as oxidation, addition of polyphenols, or in vivo dosing of diabetic patients with polyphenols.

Methods: In the present study we determined labeled rePON1 binding to HDL under various oxidative conditions by using polyacrylamide gel electrophoresis for the separation of free labeled rePON1 from HDL-bound labeled rePON1.

Results: The HDL-rePON1/free rePON1 ratio gradually decreased as the extent of HDL oxidation increased, and the antioxidants vitamin E or pomegranate juice (PJ) inhibited the redistribution of rePON1. PJ or its purified polyphenols, punicalagin, gallic acid, or ellagic acid, increased rePON1 binding also to non-oxidized HDL. Further, rePON1 associated more efficiently with HDLs isolated from diabetic patients after PJ consumption versus HDLs isolated before PJ consumption.

Conclusions: We conclude that 1) oxidative stress impairs binding of fluorescein isothiocyanate-labeled rePON1 to HDL and 2) PJ polyphenols directly increase the HDL-rePON1 association beyond their antioxidative effect. © 2010 Elsevier Inc. All rights reserved.

Keywords:

High-density lipoprotein; Paraoxonase-1; Pomegranate juice; Polyphenols; Vitamin E; Oxidative stress

Introduction

Paraoxonase genes 1, 2, and 3 are members of a multigene family [1], which were shown to be inversely related to the risk of cardiovascular diseases [2–5]. Serum paraoxonase-1 (PON1) is a high-density lipoprotein (HDL)-associated lipolactonase [6] with antioxidative properties that were associated with the enzyme's capability to protect low-density lipoprotein (LDL) [7,8] and HDL [9] from oxidation, to decrease macrophage oxidative status [10], and to attenuate atherosclerosis. PON1 antiatherogenic properties include the breakdown of oxidized lipids in oxidized lipoproteins

and macrophages [11], inhibition of oxidized LDL uptake by the cells [3], decrease in macrophage cholesterol biosynthesis [12], and stimulation of HDL-mediated cholesterol efflux from macrophages [13]. Recently, studies in PON1-knockout or PON1-transgenic mice demonstrated that PON1 has a protective role against diabetes development, secondary to its unique antioxidant properties [14]. Conversely, PON1 activity has been shown to be reduced in patients with type 2 diabetes mellitus, and in these patients, a more severe coronary artery disease was observed, as measured by coronary angiography [15]. We recently demonstrated that, in serum from diabetic patients, PON1 distributed from HDL to the lipoprotein deficient serum fraction resulted in a reduction in HDL-associated PON1 and an increase in serum free PON1 in comparison with controls. PON1 in lipoprotein-deficient serum, unlike PON1 in HDL, is less antiatherogenic, because it is less able to protect against lipid peroxidation and to stimulate macrophage

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cholesterol efflux [16]. The high concentrations of glucose in diabetic serum could account for PON1 dissociation from HDL [17]. Nevertheless, the mechanism responsible for the dissociation of PON1 from HDL in diabetic patients remains largely unknown. Because diabetes is associated with increased oxidative stress [18,19], we hypothesized that the ratio of HDL-PON1 to free PON1 would be decreased in serum from diabetic patients in comparison with controls, because of their increased serum oxidative stress, and that supplementation of antioxidants would sustain a high HDL-PON1/free PON1 ratio.

Pomegranate juice (PJ) contains polymolecular ellagitannin compounds, such as punicalagin, which are potent antioxidants [20,21]. Our recent findings showed that consumption of PJ by diabetic patients decreased oxidative stress in their serum and macrophages [22], contributed to PON1 stabilization, increased PON1 association with HDL, and stimulated enzyme catalytic activities [23]. These findings support our hypothesis that supplementation of antioxidants may sustain a high HDL-PON1/free PON1 ratio. However, the precise mechanisms involved in these effects remain elusive.

The aims of the present study were to determine if a fluorescently labeled recombinant PON1 (rePON1) could be used to assay the binding capacity of native and oxidized HDL in the presence and absence of PJ polyphenols. In the present study we determined rePON1 binding to HDL under various oxidative conditions by using a newly developed method for the separation of free rePON1 from rePON1 that is bound to HDL.

Materials and methods

Chemicals

We purchased 2,2-azobis 2-amidino-propane dihydrochloride (AAPH) from Wako, Japan. Fluorescein isothiocyanate (FITC) and vitamin E were from Sigma-Aldrich (St. Louis, MO, USA), and copper (II)-sulfate-5-hydrate (CuSO_4) was from Merck (Darmstadt, Germany).

Subjects

Six male patients with type 2 diabetes mellitus, age 59 ± 2 y, with serum glucose levels of 199 ± 33 mg/dL, hemoglobin A1c levels of 7.5–11.3%, and body mass index of 30 ± 3 kg/m², consumed PJ [50 mL of concentrated PJ/day, which contained 2600 ppm of gallic acid equivalents (GAE) polyphenols] for a period of 4 wk. Blood was collected from the patients before and after PJ consumption after 12 h of fasting. Plasma was separated by centrifugation 20 min after blood collection and was kept at 4°C for 4 wk until collection of the second plasma sample after PJ consumption. Then, the HDLs were separated as described below from all plasma samples and immediately analyzed for the binding of FITC-labeled rePON1. One HDL fraction was separated

from a plasma sample at baseline and 4 wk after storage at 4°C and served as an internal control. Both HDLs exhibited similar capacities to bind PON1, showing that storage did not contribute to detectable changes within the experimental error of the method.

The study protocol was approved by the Rambam Helsinki Committee (no. 3073). The patients served as their own controls because we compared all data after the pomegranate consumption with the baseline values. All patients were non-smokers and were treated with statins for at least 2 wk before the beginning of the study.

LDL and HDL and oxidatively modified HDL preparations

The LDL and HDL were isolated from human plasma samples by discontinuous density gradient ultracentrifugation [24] and dialyzed against saline containing sodium ethylenediaminetetra-acetic acid (1 mmol/L) to protect the lipoproteins against oxidation. Ethylenediaminetetra-acetic acid, which inactivates PON1 catalytic activity, did not interfere with the binding studies, because PON1 was quantitated by protein and not by activity. HDL protein concentration was determined with the Folin phenol reagent [25]. For oxidation, HDL was diluted in phosphate buffered saline to 2 mg of protein/mL and dialyzed overnight against phosphate buffered saline at 4°C to remove the ethylenediaminetetra-acetic acid. Oxidation of HDL was carried out at 37°C under air in a shaking water bath. HDL (2 g of protein/L) was incubated for the indicated periods of time at 37°C with the indicated concentrations of freshly prepared CuSO_4 or AAPH. Oxidation was terminated by refrigeration at 4°C. The oxidation extent of oxidatively modified HDL was determined by the thiobarbituric acid-reactive substances assay [26].

Pomegranate juice

Pomegranate juice was prepared from the whole fruit that was cut to expose arils during the squeezing process. The juice was filtered, pasteurized, concentrated to 65 Brix (Brix is a measurement of soluble solids in fruit juice and represents the sugars and many other soluble substances such as salts, acids, and tannins. Brix is measured in grams per hundred milliliters, so 65 Brix means that 100 mL of juice contains 65 g of dissolved compounds) and stored at -18°C . The concentrated PJ was diluted 1:4 (v:v) to 16 Brix with water to obtain a single-strength PJ to be used in the study. PJ was supplied by POM Wonderful (Los Angeles, CA, USA).

Determination of PJ polyphenols

Total polyphenols were determined by the method of Singleton and Rossi [27] using gallic acid as a standard and expressed as micromoles of GAE per liter.

Recombinant PON1 preparation

A recombinant PON1 variant named G3C9 (rePON1) was generated by directed evolution for expression in *Escherichia coli* [28]. A PON1 truncated variant lacking the first 20 amino acids corresponding to the hydrophobic N-terminus (Δ 20-rePON1) was constructed from the rePON1-G3C9-His8 gene by polymerase chain reaction amplification with a primer that introduced an *Nco*I site at the 5' end of the gene encoding Gln20 and recloned into pET32-trx [29]. A double histidine mutant used was H115Q/H134Q. The histidines at positions 115 and 134 were substituted with glutamine (H115Q and H134Q, respectively). The double mutant H115Q/H134Q exhibited almost no lactonase or arylesterase activity [100-fold lower compared with wild type (WT) PON1]. Purified rePON1 was stored in the storage buffer (50 mM Tris, pH 8.0; 50 mM NaCl; 1 mM CaCl₂; and 0.1% Tergitol). The rePON1 samples were delipidated using Bio-Beads SM-2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to minimize the amount of detergent present in the protein samples and to increase rePON1 binding to HDL.

rePON1 labeling with FITC

Recombinant PON1 was conjugated to FITC. Briefly, rePON1 (1 mL containing 2 mg of protein) was dialyzed overnight at 4°C against several changes of borate buffer (0.1 M borate, 25 mM sodium tetraborate, 75 mM NaCl, pH 8.6). FITC was dissolved in dimethyl formamide to a stock solution of 10 mg/mL and 20 mL of FITC was added drop-wise to 1 mL of rePON1 (2 mg protein/mL) to obtain a final concentration of 0.2 mg of FITC/mL, followed by an incubation for 1 h at room temperature with constant stirring. FITC-conjugated rePON1 (FITC-rePON1) was separated from unconjugated FITC by size exclusion chromatography over a PD-10 column (Amersham-Pharmacia Biotech, Uppsala, Sweden) using an elution solution of 10 mM phosphate buffer, pH 8.0.

Incubation procedure

Non-oxidized and oxidized HDLs (1.4 mg of protein/mL) were incubated with FITC-rePON1 (0.7 mg protein/mL) in a final volume of 22 μ L for 1 h at 37°C. The amount of native PON1 in the HDLs used was 32 ± 5.8 μ g of protein/mg of HDL protein, and the variations among different HDL preparations did not significantly affect the binding of FITC-rePON1. Then, each sample was mixed with a sample buffer (62.5 mM Tris-HCL, pH 6.8, 80% glycerol, 0.01% bromophenol blue) and subjected to polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

Samples were loaded on 10% polyacrylamide Tris-HCL gel (precast gel for polyacrylamide electrophoresis; Bio-Rad). Running conditions were 200 V at 100–60 mA for

35 min. HDL-associated FITC-rePON1 produces a clear band at the upper side of the gel, whereas the free FITC-rePON1 runs to the lower part of the gel, resulting in a clear separation between HDL-bound rePON1 and free rePON1 (which did not bind to HDL). The bands were visualized and densitometric analyses were performed using Phosphor Imager (Fluorescent Image Analysis FLA-2000, Fujifilm, Tokyo, Japan).

Statistical analyses

Each experiment was performed in triplicate, and each experiment was separately performed three times ($n = 3$). Statistical analyses used one-way analysis of variance followed by the Student-Newman-Keuls test for comparing differences among multiple groups. Results are expressed as mean \pm standard deviation (SD) of three separate experiments.

Results

Increasing concentrations of FITC-rePON1 were applied to polyacrylamide gel electrophoresis. Figure 1A demonstrates that lipoprotein free FITC-rePON1 can be visualized in the polyacrylamide gel in a dose-dependent manner. Based on these results, we next incubated increasing concentrations of FITC-rePON1 for 1 h at 37°C with HDL. Figure 1B shows that FITC-rePON1 binds in part to HDL, and the remaining free FITC-rePON1 can be visualized at the bottom of the gel. Densitometric analyses demonstrated that the measured fluorescence of the free rePON1 (Fig. 1C) and of the HDL-bound rePON1 (Fig. 1D) gradually increased in a linear manner, and the calculated HDL-rePON1/free rePON1 ratio remained constant (Fig. 1E). In contrast, migration of FITC-rePON1 in the gel in the presence of LDL confirmed previous reports that PON1 does not bind to LDL (Fig. 1F), showing that the binding of rePON1 is specific to HDL.

Based on these results, in the following incubation studies we used rePON1 at a concentration of 0.7 mg of protein/mL.

Incubation of HDL (2 g of protein/L) for 2 h at 37°C with increasing concentrations of freshly prepared CuSO₄ or with the free radical generator AAPH gradually increased the extent of HDL oxidation, as determined by the thiobarbituric acid-reactive substances (Fig. 2A) and lipid peroxide (Fig. 2B) assays. The non-oxidized and oxidized HDLs were incubated with FITC-rePON1 for 1 h at 37°C. Figure 2C shows that the HDL-rePON1/free rePON1 ratio gradually and significantly ($P < 0.01$) decreases as HDL oxidation extent increases, suggesting that oxidative stress, induced by copper ions or by AAPH-generated free radicals, impairs the binding of rePON1 to HDL. Figure 2D illustrates a typical example of one gel electrophoresis pattern.

To confirm that rePON1 indeed binds to HDL, we incubated HDL with an FITC-rePON1 catalytic histidine dyad double mutant (H115Q/H134Q), which lacks catalytic activity, and an FITC-rePON1 mutant lacking the 20 amino acids

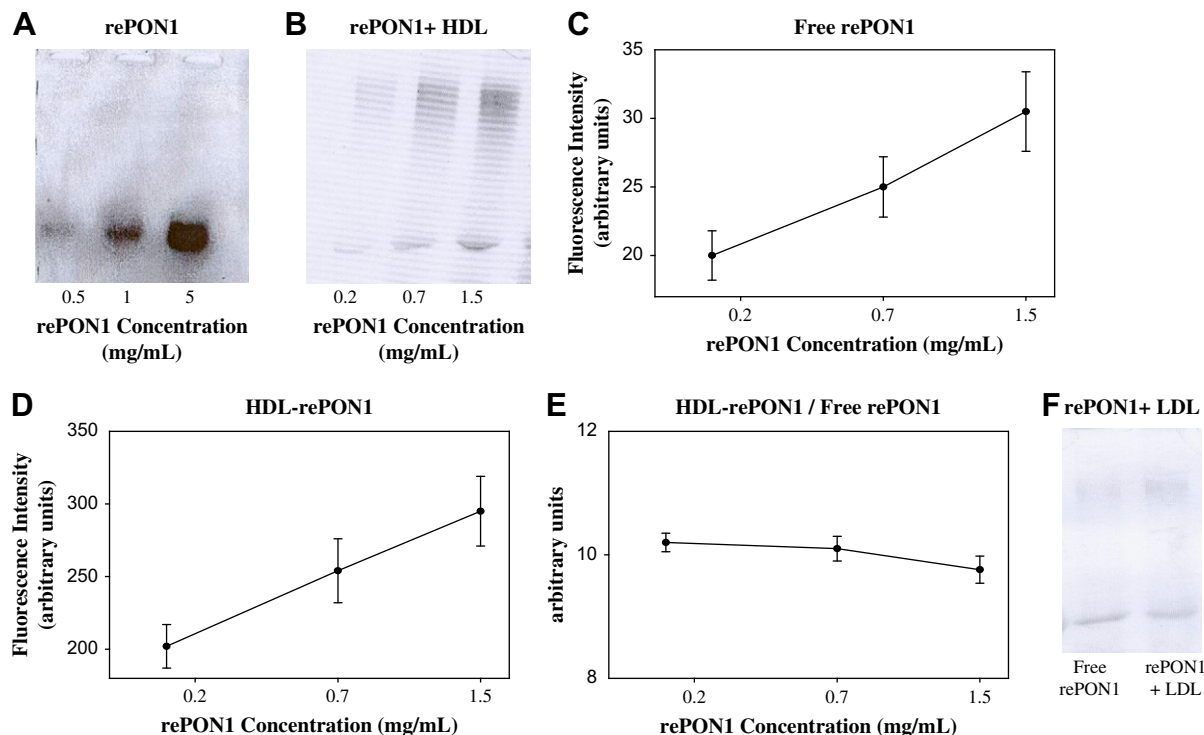


Fig. 1. Separation of HDL-associated rePON1 versus free rePON1 by electrophoresis on polyacrylamide gels. (A) Free rePON1 concentration curve. (B) HDL was incubated with increasing concentrations of fluorescein isothiocyanate–conjugated rePON1 as described in MATERIALS AND METHODS, and HDL-associated rePON1 was separated from free rePON1 by electrophoresis on a polyacrylamide gel. (C–E) Concentration curves of free rePON1, HDL-rePON1, and HDL-rePON1/free rePON1 ratio. Results represent mean \pm SD of three separate experiments. (F) Fluorescein isothiocyanate–conjugated rePON1 was incubated with LDL and LDL-associated rePON1 was separated from free rePON1 by electrophoresis on a polyacrylamide gel. HDL, high-density lipoprotein; LDL, low-density lipoprotein; rePON1, recombinant paraoxonase-1.

from the N-terminal region (PON1- Δ 20), which lacks HDL-binding ability [17,30]. The HDL-rePON1/free rePON1 ratio on using the H115Q/H134Q rePON1 was similar to that of rePON1 (2.98 ± 0.2 and 2.8 ± 0.17 , respectively). In contrast, on incubation of HDL with FITC-conjugated Δ 20-PON1, the HDL-rePON1/free rePON1 ratio decreased by 4.5-fold, to 0.66 ± 0.05 . These results support the reliability of free rePON1 separation from HDL-associated rePON1 by polyacrylamide gel electrophoresis.

To confirm that the oxidative stress, and not the oxidizing compounds themselves, affect the binding of rePON1 to HDL, HDL was oxidized with AAPH (20 mmol/L) or with CuSO_4 (40 $\mu\text{mol/L}$) in the absence or presence of increasing concentrations of the antioxidant vitamin E (0–250 $\mu\text{mol/L}$) or PJ (0–160 μmol of GAE/L). The extent of HDL oxidation, measured as thiobarbituric acid-reactive substances, gradually decreased in a vitamin E or PJ dose-dependent manner (Fig. 3A,C). In parallel, the reduced capacity of oxidatively modified HDL to bind PON1 was gradually restored, and these effects were more evident when HDL was oxidized with CuSO_4 than with AAPH (Fig. 3B,D). Nevertheless, even the highest concentration of vitamin E used (250 $\mu\text{mol/L}$) did not fully restore the capacity of HDL to bind rePON1 to the values obtained with non-oxidized HDL. On the contrary, when high PJ con-

centrations were used (40 and 160 μmol of GAE/L), rePON1 binding to copper ion-oxidized HDL significantly ($P < 0.01$) increased over the values obtained for the control non-oxidized HDL. These results could suggest that PJ polyphenols, in addition to their beneficial effect on HDL-rePON1 association by their antioxidant property, may also directly exhibit a possible bridging stabilizing effect on HDL-rePON1 binding.

Next, we analyzed the effect of PJ and its purified polyphenolic components on the association of HDL with rePON1 under non-oxidative conditions. Punicalagin, gallic acid, and ellagic acid, which are found in abundance in PJ, are potent antioxidants, because they were found to inhibit CuSO_4 -induced LDL oxidation with a concentration needed to inhibit LDL oxidation by 50% of 5.85, 4.26, and 10.64 $\mu\text{mol/L}$, respectively. Incubation of HDL (non-oxidized HDL) with rePON1 in presence of increasing concentrations of PJ remarkably ($P < 0.01$) increased the HDL-rePON1/free rePON1 ratio by up to 64% (Fig. 4A). Similarly, punicalagin, gallic acid, and ellagic acid increased the HDL-rePON1/free rePON1 ratio in a dose-dependent manner. However, punicalagin exhibited a most remarkable effect, because at a concentration of 8 $\mu\text{mol/L}$ it increased rePON1 binding to HDL by 63%, whereas at this concentration gallic and ellagic acids

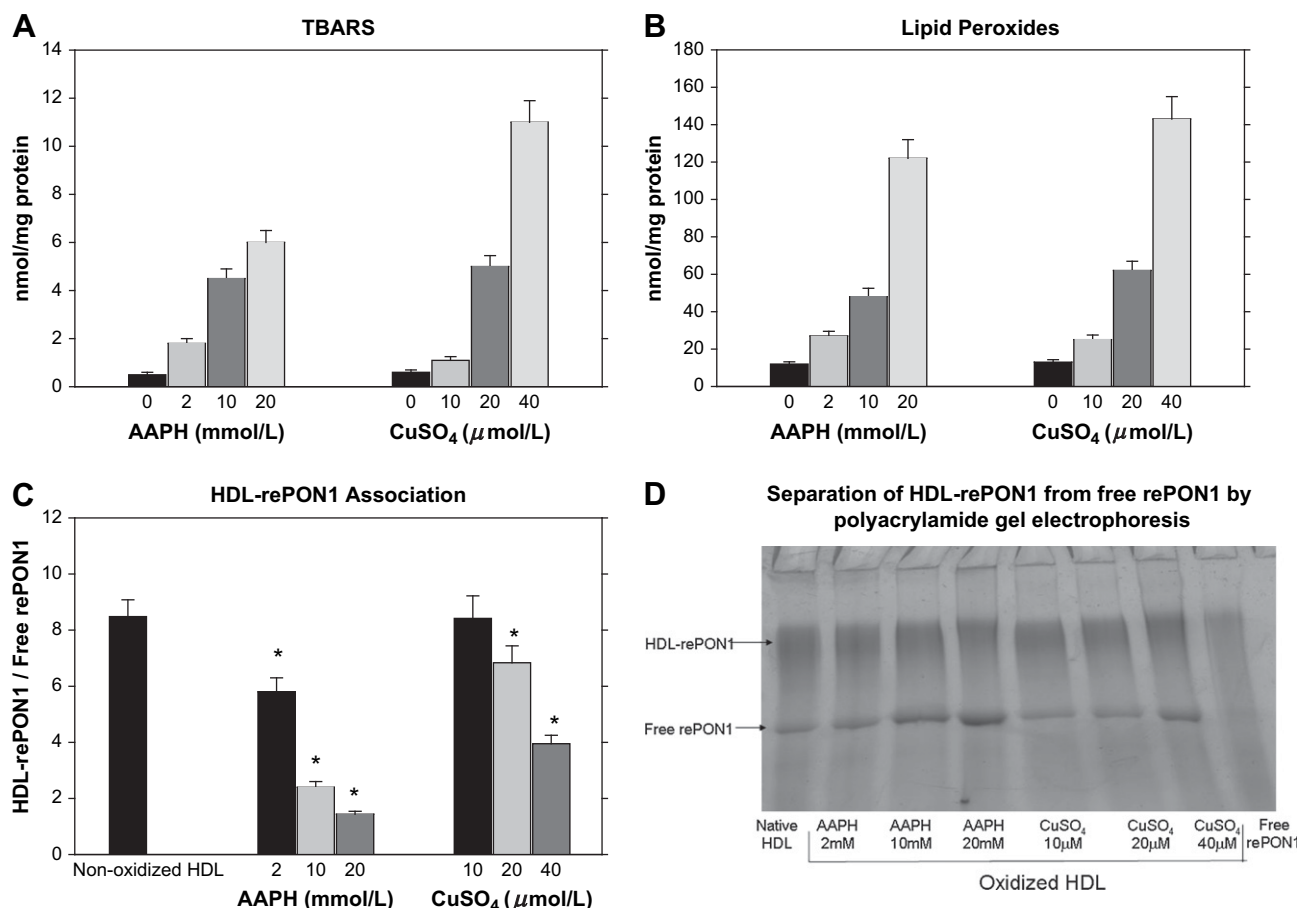


Fig. 2. Oxidative stress reduces the association of rePON1 with HDL. HDL (2 g of protein/L) was incubated for 2 h at 37°C with increasing concentrations of freshly prepared CuSO₄ or AAPH. HDL oxidation was determined by the (A) TBARS and (B) lipid peroxide assays. Oxidized HDLs were incubated with fluorescein isothiocyanate–conjugated rePON1 as described in MATERIALS AND METHODS, and HDL-associated rePON1 versus free rePON1 was determined by electrophoresis on a polyacrylamide gel. Non-oxidized HDL served as control. (C) Mean \pm SD for the HDL-rePON1/free rePON1 ratio ($n = 3$ separate experiments). * $P < 0.01$ versus non-oxidized HDL. (D) A gel running. AAPH, 2,2-azobis 2-amidino-propane dihydrochloride; CuSO₄, copper (II)-sulfate-5-hydrate; HDL, high-density lipoprotein; rePON1, recombinant paraoxonase-1; TBARS, thiobarbituric acid-reactive substances.

increased rePON1 binding to HDL by only 10% and 19%, respectively. Maximal 47%, and 34% increases in rePON1 binding to HDL were obtained when using four-fold higher concentrations (32 μ mol/L) of gallic and ellagic acids, respectively (Fig. 4B). These results confirm that PJ and its purified polyphenols have a direct effect on rePON1 binding to HDL, which is beyond their antioxidative capacities, and that punicalagin is the most potent one in this respect.

To extend these findings to in vivo physiologic relevance, we examined the association of rePON1 with HDL isolated from six diabetic patients before and after consumption of PJ. Three healthy subjects served as controls. Diabetic patients are characterized by increased oxidative stress [18], and their HDL oxidation is higher than in controls [31]. The HDLs were incubated with FITC-rePON1 for 1 h at 37°C. The HDL-rePON1/free rePON1 ratio in the control subjects was 10.3 ± 1.4 (mean \pm SD), whereas in the diabetic patients before PJ consumption the HDL-rePON1/free rePON1 ratio significantly ($P < 0.05$) decreased to

8.3 ± 2.1 (mean \pm SD), suggesting a lower capacity of HDL from diabetic patients to bind rePON1. However, when using HDL from diabetic patients after the consumption of PJ, the HDL-rePON1/free rePON1 ratio increased to 11.6 ± 2.9 (mean \pm SD). Figure 5 demonstrates the HDL-rePON1/free rePON1 ratio for each of the diabetic patients using HDL derived before or after PJ consumption. These results confirm that PJ increases the capacity of HDL to bind rePON1 not only in vitro, but also in vivo.

Discussion

The present study demonstrates, for the first time, that it is possible to separate HDL-associated rePON1 from free rePON1 (not associated with lipoprotein) by a simple polyacrylamide gel electrophoresis technique. By using this method, we showed that, under conditions of oxidative stress, the binding of rePON1 to HDL is significantly decreased. Antioxidants such as vitamin E and, more so, PJ polyphenols increase the capacity of HDL to bind rePON1, as shown in

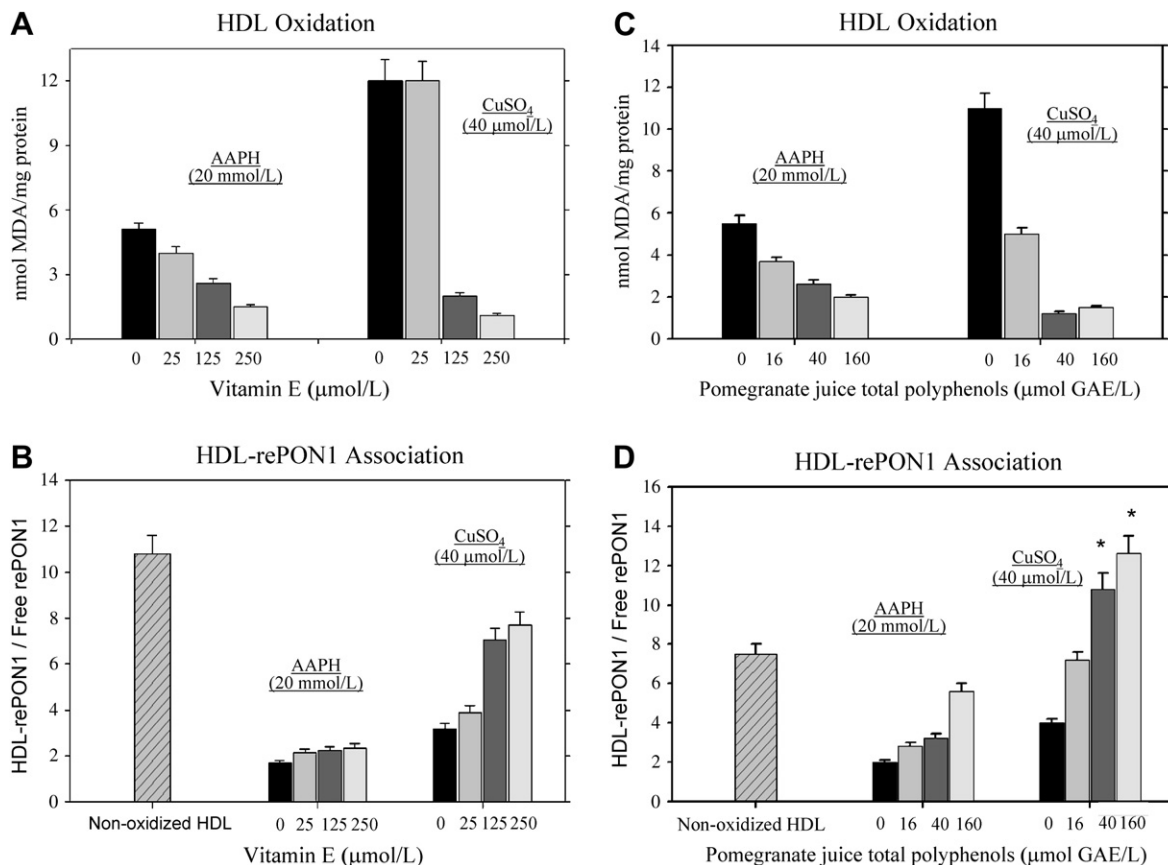


Fig. 3. The rePON1 association with HDL under oxidative stress: effect of vitamin E and pomegranate juice. HDL was oxidized with AAPH (20 mmol/L) or with CuSO_4 (40 $\mu\text{mol/L}$) in the absence or presence of increasing concentrations of vitamin E (0–250 $\mu\text{mol/L}$) or pomegranate juice (0–160 $\mu\text{mol GAE/L}$). (A, C) Extent of HDL oxidation was measured as thiobarbituric acid-reactive substances. Oxidized HDLs were incubated with fluorescein isothiocyanate-conjugated rePON1 as described in MATERIALS AND METHODS, and HDL-associated rePON1 versus free rePON1 was determined by electrophoresis on a polyacrylamide gel. Effects of (B) vitamin E and (D) pomegranate juice are shown. Non-oxidized HDL served as a control. Results are expressed as mean \pm SD for the HDL-rePON1/free rePON1 ratio ($n = 3$ separate experiments). * $P < 0.01$ versus non-oxidized HDL. AAPH, 2,2-azobis 2-amidino-propane dihydrochloride; CuSO_4 , copper (II)-sulfate-5-hydrate; GAE, gallic acid equivalents; HDL, high-density lipoprotein; rePON1, recombinant paraoxonase-1; MDA, malonaldehyde.

vitro and in vivo in diabetic patients after consumption of PJ. These effects could be related to a PJ-mediated reduction in oxidative stress and to a direct effect of PJ polyphenols on a HDL-PON1 association.

We demonstrated recently that consumption of PJ or extracts by diabetic patients increased a HDL-associated PON1 in serum and decreased PON1 in lipoprotein-deficient serum [22, 23]. Furthermore, addition of the pomegranate-derived polyphenol, punicalagin, to serum from diabetic patients before HDL separation by ultracentrifugation increased the HDL-bound PON1 protein, as shown by western blot analysis. These effects could have resulted from an increased binding or a decreased dissociation of PON1 to/from HDL. In the present study we have extended these findings to show that PJ and its purified polyphenols directly increase the binding of rePON1 to HDL. This clear evidence was obtained by employing a new method for separation of HDL-associated rePON1 from free non-HDL-associated rePON1 by polyacrylamide gel electrophoresis. By incubation of HDL with FITC-rePON1, we could follow and record the fluorescence of HDL or of the free rePON1 that migrated to different po-

sitions. Oxidative stress induces modifications in the HDL particle, such as the formation of aldehydes, as a result of polyunsaturated fatty acids oxidation. Oxidation of amino acids, such as methionine and tryptophan, could play important roles in the conformation and function of apolipoprotein A-I and, consequently, the binding of rePON1 to HDL. This finding may explain the reduced HDL-associated PON1 that we previously observed in serum from diabetic patients (characterized by increased oxidative stress). The present study further confirms these reports, because we found a significant reduced ability of HDL from diabetic patients to bind rePON1 in comparison with HDL from healthy controls. However, the tests were done on a small number of subjects, and to power these results larger studies are needed in the future. Furthermore, these results cannot exclude the possibility that oxidative stress also dissociates PON1 from HDL, and probably both effects contribute simultaneously to the binding behavior of rePON1.

Decreased HDL-PON1 binding capacity was further demonstrated in the present study when using HDL particles isolated from diabetic patients, before and after PJ consumption.

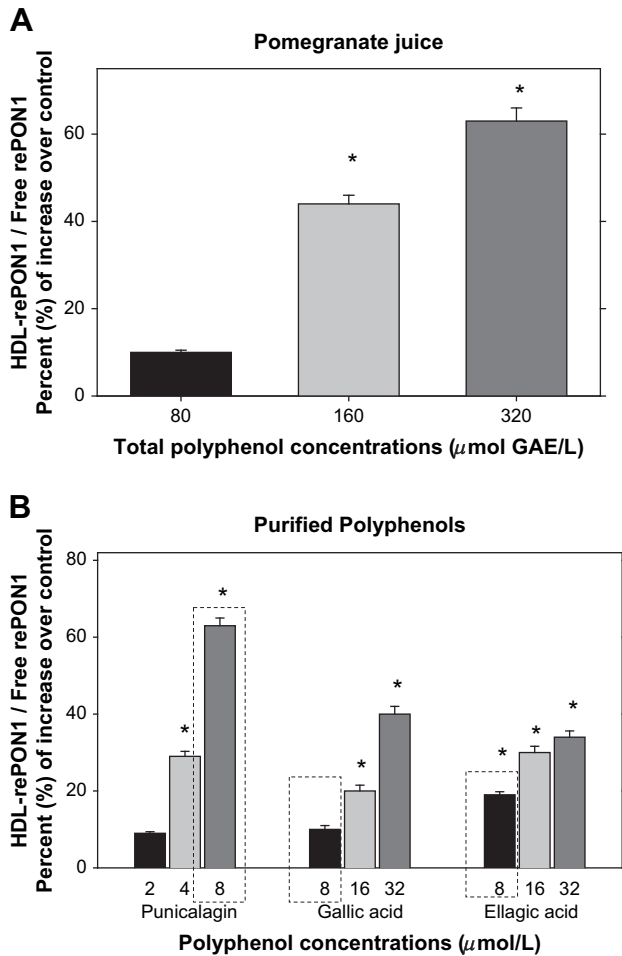


Fig. 4. Pomegranate juice polyphenols increase the rePON1 association with HDL. HDL (non-oxidized) was preincubated for 1 h at 37°C with (A) increasing concentrations of pomegranate juice (80, 160, and 320 µmol GAE/L) or (B) increasing concentrations of the purified polyphenols punicalagin (2, 4, and 8 µmol/L) or gallic acid and ellagic acid (8, 16, and 32 µmol/L), and then incubated with fluorescein isothiocyanate-conjugated rePON1 as described in MATERIALS AND METHODS. HDL-associated rePON1 versus free rePON1 was determined by electrophoresis on a polyacrylamide gel. Results are expressed as mean \pm SD for the HDL-rePON1/free rePON1 ratio ($n = 3$ separate experiments). * $P < 0.01$ versus control. GAE, gallic acid equivalents; HDL, high-density lipoprotein; rePON1, recombinant paraoxonase-1.

These lipoproteins were incubated *ex vivo* with FITC-rePON1, and the present results clearly show that PJ consumption increased the capacity of the HDL to bind rePON1. These results can be related to the reduction in patients' serum oxidative stress after PJ consumption, along with a direct beneficial effect of PJ polyphenols on HDL-PON1 binding capacity. This capability of PJ polyphenols to increase the binding of rePON1 to HDL, however, was also shown *in vitro*. HDL, which was oxidized in the presence of PJ, exhibited an increased capacity to bind rePON1, which was significantly increased over the capacity of non-oxidized HDL to bind rePON1. However, this effect was evident only when HDL was oxidized with copper ions, but not with AAPH.

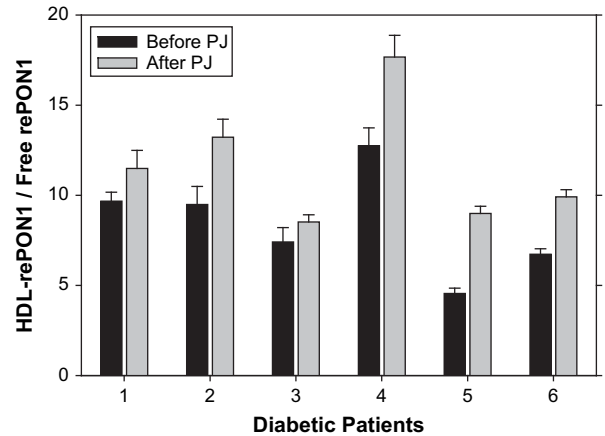


Fig. 5. The rePON1 association with HDL derived from diabetic patients: effect of PJ consumption. HDL was isolated from plasma samples of six diabetic patients taken before and after PJ consumption. The HDLs were incubated with fluorescein isothiocyanate-conjugated rePON1 and the HDL-rePON1/free rePON1 ratio was determined as described in MATERIALS AND METHODS. The results are expressed as mean \pm SD of three separate determinations of the individual HDL-rePON1/free rePON1 ratio using HDL derived before and after PJ consumption. HDL, high-density lipoprotein; rePON1, recombinant paraoxonase-1; PJ, pomegranate juice.

This discrepancy can be related to a different mode of action of copper ions and AAPH. Although AAPH was shown to generate free radicals at a constant rate at a certain temperature, with a random attack on the endogenous constituents of the lipoprotein [32], copper ion-induced LDL oxidation requires the presence of preformed lipid hydroperoxide and generates different oxidized constituents [33]. Thus, it is possible that PJ interacts with a specific oxidized product formed as a consequence of HDL oxidation by copper ions, but not by AAPH. Furthermore, polyphenols are known to inhibit LDL/HDL oxidation by different mechanisms. They may donate a hydrogen atom and thus reduce the generated free radical, and/or they may chelate metal ions, such as iron or copper. In this case, in the system of HDL oxidation by copper ions, the PJ polyphenols will exert radical scavenging and chelation of metal ions, whereas in the AAPH-induced oxidation, only the PJ-mediated radical scavenging mechanism will play a role.

The experimental design of this study cannot distinguish between effects of the test compounds on the oxidation reaction separately from the effects of the test compounds themselves on the biologic matrix that PON1 is binding to. However, Figure 4 shows that incubation of non-oxidized HDL with rePON1 in the presence of PJ polyphenols remarkably and significantly increased the HDL-PON1 interaction. This result may point to a possible direct effect of polyphenols between rePON1 and HDL, which is beyond the antioxidative effects of these compounds. It is thus possible that PJ polyphenols serve as bridging molecules that enhance the binding of free rePON1 to HDL. Further studies are needed to directly elucidate this phenomenon.

In summary, the present study shows the following new findings: 1) HDL-associated rePON1 can be separated from

free rePON1 by a polyacrylamide gel electrophoresis technique; 2) oxidative stress impairs binding of rePON1 to HDL; and 3) PJ polyphenols increase the capacity of HDL to bind rePON1 in vitro and in vivo. These effects could be related to PJ-mediated reduction in oxidative stress and to a direct effect of PJ phenolics on the HDL-rePON1 association.

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