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Enzymatic assessment of paraoxonase 1 activity on HDL subclasses: A practical zymogram method to assess HDL function

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ABSTRACT

Background: We developed a practical method for analysis of PON-1 enzymatic activity in HDL subclasses.
Methods: The assay uses 4–12% polyacrylamide gradient gels, phenylacetate as substrate coupled with densitometric phenol detection using 4-aminoantipyrine. The measurement PON-1 activity in situ across the HDL subclasses has a strong correlation with the kinetic microplate assay for total PON-1 activity, $r = 0.91$, $p < 0.001$.
Results: The same HDL-C level, healthy subjects ($n = 33$) display a large difference in the ratio of PON-1 activity in small vs. large HDL. Since PON-1 activity is larger in HDL_3 we propose that this difference has a potent predictive value for clinical risk assessment and therapeutic choice. Our method also offers the advantage of assessing the distribution of up to six different HDL apolipoproteins in the same gel after transfer.
Conclusions: We seek to further dissect the cause of a different distribution of PON-1 activity in HDL subclasses by employing this method that permits practical, inexpensive analysis of antioxidant function of HDL subclasses and has the potential for application in clinical chemistry and to shed some light on the importance of PON-1 distribution.

1. Introduction

Paraoxonase 1 (PON1) is an esterase and lactonase carried by high-density lipoprotein (HDL), and is known as one of the antioxidant system-related enzymes [1–4]. PON1 hydrolyzes lipoprotein-associated peroxides and lactones [1–6]. PON1 exerts some of its physiological functions by eliminating potent oxidants and/or by neutralizing their end-products to non-toxic moieties [7–11]. Epidemiological studies have shown a negative correlation between HDL-cholesterol (HDL-C) and the development of coronary heart disease (CAD). The causal relationship between plasma/serum HDL-C concentration and CAD has been explained by the role played by these lipoproteins in reverse cholesterol transport, as well as by other potentially anti-atherogenic properties of HDL, such as their anti-inflammatory, anti-oxidative, antiplatelet, anti-coagulant, and pro-fibrinolytic effects [12–18]. However, recent clinical trials [19] and Mendelian randomization studies [20] do not support a role in increasing HDL-C levels as a protective measure against coronary artery disease (CAD). This present controversy emphasizes the need for the development of functional techniques to explore HDL functions that are practical and adapted to clinical studies and eventually that may become useful in clinical pathology. Such methods exist for dysfunctional HDL but not for PON1 [21,22].

Several studies have shown that discrete subclasses of HDL bear different patterns of proteins [15–18,23–28]. Earlier studies using ultracentrifugation have provided evidence for PON1 activity residing preferentially in the smaller HDL_3 particles [29]. More recent proteomic studies have confirmed these findings and added the evidence that structural, and more importantly, functional proteins in HDL tend to cluster in particles with different functional properties [15–18]. HDL_3 particles can be more potent antioxidants, in part due to their PON1 content.

Notably, a recent study shows that HDL isolated from patients with CAD has compromised antioxidant and endothelial protective activities [30]. This finding has been associated with decreased PON1 activity in small HDL particles correlated with their modification by malondialdehyde [30].

HDLs encompass a heterogeneous group of lipoproteins that may be classified by increasing size in HDL_3c, HDL_3b, HDL_3a; HDL_2b, and HDL_2a, as measured by native PAGE [11]. HDL subclasses are currently assessed and have been classified by other approaches, such as selective precipitation ultracentrifugation, nuclear magnetic resonance (NMR), electronic microscopy, and 2-dimensional electrophoresis [11]. All these approaches distinguish HDL particles of different sizes or densities

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that might have different atheroprotective properties. However, the most sophisticated methods available today offer information on particle size and number; protein or lipid content yet none informs on any functional property of HDL. Which HDL fraction confers better cardiovascular protection remains controversial. It has been postulated that the large HDL fraction is the most atheroprotective, because CAD patients have lower levels of these particles than controls do, as assessed by selective precipitation or NMR [11,12]. In contrast, as stated above small HDL particles are the best acceptors of cholesterol from peripheral tissues and also have better antioxidant properties than large HDL particles [11,14–18]. Moreover, thiazolidinediones as well as fibrates, both antiatherogenic drugs that increase HDL-C levels, shift HDL size distribution toward small HDL particles [13]. In addition, some subjects with severe hypo-alphalipoproteinemia or apoAI Milano who do not develop CAD have a high proportion of small HDL suggesting an atheroprotective role of these particles [13]. Small HDL particles are protein rich and lipid poor, as opposed to large particles; therefore, the relative proportion of HDL subclasses is dependent on the component determined for the quantification [11,13–18]. The broad diversity of methods used for determining HDL subclasses is partly responsible for the apparent controversy concerning which is the most antiatherogenic fraction of HDL.

We hypothesize that independently of HDL-C concentrations or particle number one of the factors that may contribute to CAD is poor function of PON1 in HDL_3 or its redistribution to larger HDL particles. To test this hypothesis, the need for a practical method to quantify PON1 activity in HDL subclasses becomes apparent. Therefore, with the long-term goal of elucidating possible differences in PON1 function in HDL subclasses, we have first developed a method that combines native gradient gel electrophoresis and PON1 activity measurement in the same gel. Our method, therefore, allows for simultaneous analysis of HDL subclasses distribution in patients' sera and of the PON1 enzymatic antioxidant activity in each fraction.

2. Materials and methods

2.1. Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO).

2.2. Study subjects

Volunteers from the staff of Kyoto Medical Center (hospital workers that received their annual health check up) were recruited. All participants were in good health, not current smokers, not on any medication and had not been diagnosed to have diabetes mellitus nor cardiovascular, liver, kidney, nutritional or collagen disorders. From this population we chose 33 subjects to have a range of HDL-C from 0.6 to 3.6 mmol/l. For the body mass index (BMI) the weight was measured in light indoor clothing without shoes. The systolic and diastolic blood pressure was measured in the right arm of each participant in the seated position using the sphygmomanometer. The study was approved by the ethics committee of Kyoto Medical Center and each subject gave informed consent.

2.3. General laboratory assessments

Blood samples were obtained in dry tubes after overnight fasting from the antecubital vein. Samples were centrifuged at 4 °C, and serum was separated and analyzed or frozen at −80 °C until analysis. Plasma glucose and serum lipids, such as low-density lipoprotein (LDL) cholesterol (LDL-C), HDL cholesterol (HDL-C) and triglycerides (TG), were measured using enzymatic methods.

2.4. Isolation of HDL

HDLs were separated by ultracentrifugation of pooled serum in a Beckman L8-70 M ultracentrifuge at 110,000 rpm in 8.4 ml polycarbonate tubes in a 50 Ti rotor as described previously [31]. Briefly, total apo B-containing lipoproteins (density < 1.063 mg/dl) were separated by 20 h centrifugation, then total HDL (density 1.063–1.21 g/ml) was obtained after 24 h centrifugation and repurified as HDL_2 and HDL_3 (density 1.12 and 1.21, respectively). HDLs were dialyzed against phosphate buffered saline containing antiproteases and 1 mmol/l ascorbic acid.

2.5. PON1 activity

Serum PON1 arylesterase activity was kinetically measured using phenylacetate as a substrate at 37 °C, and the absorbance changes were recorded at 270 nm in a Versamax Microplate Reader (Molecular Devices, Sunnyvale CA), as described previously [2,9,31]. The PON1 lactonase activity was kinetically measured using DHC as a substrate at 37 °C, and recorded at 270 nm in a Versamax Microplate Reader as described previously [2,9,31].

2.6. PON1 activity detection in situ

2.6.1. Buffers/reagents

The following buffers and reagents were used: Native running buffer: 25 mmol/l Tris, 192 mmol/l glycine pH 8.3. 2 × sample buffer: 62.5 mmol/l Tris HCl, pH 6.8 and 25% glycerol. 0.01% bromophenol blue was used for molecular weight/size standards. Reaction buffer: 50 ml 0.1 mol/l Tris pH 8.3 1 mmol/l $CaCl_2$. Substrate: 1 mmol/l phenylacetate in reaction buffer. Developing reagent: 1 mol/l aminoantipyrine (4-AAP) and 1 mol/l $K_3Fe(CN)_6$. These compounds are light sensitive and should be stored in the dark. They should be prepared fresh or no more than 2 or 3 days old.

2.6.2. Electrophoresis of HDL

HDLs were separated by their hydrodynamic diameter in an 8 × 10 × 0.15 cm non-denaturing 4–12% gradient polyacrylamide gel electrophoresis (Novex ® 4–12% Tris-Glycine gel, Invitrogen, Carlsbad, CA). Serum samples were diluted 1 to 1 in 2 × sample buffer and up to 16 μl of sample are loaded. The gels were run on an XCell Sure Lock electrophoresis cell (Novex®, Invitrogen), were stacked for 30 min at 65 V and then run for 16 h at 100 V at 4 °C. In these electrophoretic conditions, lipoproteins of larger size than HDL either did not penetrate the gel or were found in the upper 1/4 of the running distance, the rest of the gel allowed for separation of HDL different subclasses in the appropriate range of 7–12 nm particle diameter. For HDL, 25 μg of protein per well was loaded.

2.6.3. Choice of substrate of PON1

When we tested paraoxon, pNPA and TBBL, the reaction is visible but difficult to assess due to the yellow product in the former 2 and to low sensitivity in the latter. Moreover, paraoxon is toxic and the activity against it varies widely with the Q192R polymorphism. Phenylacetate, however, is a polymorphism-neutral substrate, widely employed and closely reflects PON1 protein concentration. Phenol, its product, is usually detected at 270 nm. In the search for a colorimetric method that can be easily employed in clinical laboratories, we took advantage of the classic Trinder reaction to stoichiometrically detect phenol in our gels. After a wide range of concentrations and incubation conditions were tried, we reported the results of the method as we optimized it through these multiple experiments.

2.6.4. Enzymatic reaction

The gels were equilibrated in reaction buffer, 50 ml 0.1 mol/l Tris, pH 8.3, 2 mmol/l $CaCl_2$, 2 × 15 min. At this point the reaction needs to