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Macrophage paraoxonase 1 (PON1) binding sites

Michal Efrat, Michael Aviram *

The Lipid Research Laboratory, Technion Faculty of Medicine, The Rappaport Family Institute for Research in the Medical Sciences and Rambam Medical Center, Efron 1, Bat Galim, Haifa 31096, Israel

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ABSTRACT

Paraoxonase 1 (PON1), an HDL-associated esterase, is known to possess anti-oxidant and anti-atherogenic properties. PON1 was shown to protect macrophages from oxidative stress, to inhibit macrophage cholesterol biosynthesis, and to stimulate HDL-mediated cholesterol efflux from the cells. The aim of the present study was to characterize macrophage PON1 binding sites which could be responsible for the above anti-atherogenic activities.

Incubation of FITC-labeled recombinant PON1 with J774 A.1 macrophage-like cell line at 37 °C, resulted in cellular binding and internalization of PON1, leading to PON1 localization in the cell's cytoplasm compartment. In order to determine whether PON1 uptake is mediated via a specific binding to the macrophage, FITC-labeled recombinant PON1 was incubated with macrophages at 4 °C, followed by cell membranes separation. Macrophage membrane fluorescence was shown to be directly and dose-dependently related to the labeled PON1 concentration. Furthermore, binding assays performed at 4 and at 37 °C, using labeled and non-labeled recombinant PON1 (for competitive inhibition), demonstrated a dose-dependent significant 30% decrement in labeled PON1 binding to the macrophages, by the non-labeled PON1. Similarly, binding assays, using labeled PON1 and non-labeled HDL (the natural carrier of PON1 in the circulation) indicated that HDL decreased the binding of labeled PON1 to macrophages by 25%. Unlike HDL, LDL had no effect on labeled PON1 binding to macrophages. Finally, HDL were pre incubated without or with PON1 or apolipoprotein AI (apoAI) antibodies, in order to block PON1 or apoAI ability to bind to the cells. HDL incubation with antibody to PON1 or to apoAI significantly decreased HDL ability to inhibit macrophages-mediated LDL oxidation (by 32% or by 25%, respectively). A similar trend was also observed for HDL-mediated cholesterol efflux from macrophages, with an inhibitory effect of 35% or 19%, respectively. These results suggest that blocking HDL binding to macrophages through its apo A-I, and more so, via its PON1, results in the attenuation of HDL-PON1 biological activities.

In conclusion, PON1 specifically binds to macrophage binding sites, leading to anti-atherogenic effects. Macrophage PON1 binding sites may thus be a target for future cardio protection therapy.

Macrophage cholesterol accumulation and foam cell formation are the hallmark of early atherogenesis [1,2]. Cellular cholesterol accumulation can results from increased uptake of LDL or its modified forms [3,4] from decreased cellular cholesterol efflux [5], and/or from enhanced cellular cholesterol biosynthesis [6].

PON1 an HDL-associated enzyme, possesses anti-atherogenic properties including protection of LDL, HDL, and macrophages against oxidative stress [7], attenuation of oxidized-LDL uptake by macrophages [8], inhibition of macrophages cholesterol biosynthesis [9], and stimulation of HDL-mediated cholesterol efflux from the cells [10]. PON1 was shown to exhibit a wide range of hydrolytic activities such as arylesterase, phosphotriesterase, and lactonase (which best resemble PON1, physio/pathological functions [11]). Human serum PON1 activity was shown to be inversely related to the risk of cardiovascular diseases [12] and low PON1 activities were observed in atherosclerotic, hypercholesterolemic, and diabetic patients [13], as well as in the atherosclerotic apolipoprotein E-deficient (E^0) mice [14], and in rabbits fed atherogenic diet [15]. The role of PON1 in atherosclerosis development was demonstrated in studies using mice lacking PON1 [16,17], or over expressing PON1 [18].

Since PON1 was shown to interact with macrophages and these interactions have anti-atherogenic consequences, we sought analyze this interaction; i.e. the location of PON1 in the cell, specific PON1 binding sites on macrophages, and the consequent of PON1 specific binding to macrophages on PON1 biological activities.

* Corresponding author. Fax: +972 4 8542130.
E-mail address: aviram@tx.technion.ac.il (M. Aviram).

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Materials and methods

PON1 preparation

Recombinant PON1 (rePON1). Recombinant PON1 (rePON1) was generated in *Escherichia coli* by a directed evolution process as previously described [19]. PON1 storage buffer [50 mM Tris (pH 8.0), 50 mM NaCl, 1 mM $CaCl_2$, and 0.1% tergitol] was supplemented with 0.02% sodium azide and stored at 4 °C.

HDL preparation

HDL fractions were prepared from the serum of fasted normolipidemic human volunteers by discontinuous density gradient ultracentrifugation [20]. The HDLs were then dialyzed against 150 mM NaCl and 1 mM CaCl2 (pH 7.4), and their protein content was determined with the Folin phenol reagent [21].

Confocal microscopy analysis of macrophage incubated with recombinant PON1

Cells were grown on 1.5 mm cover-slide. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Permeabilized cells were stained directly with rabbit anti-mouse PON1(generous gift from Prof. Dan Tawfik, 1:100) followed by Donkey anti-rabbit antibody(Jackson Immuno Research,1:100) conjugated to Cy-2. Propidium iodide (Sigma, 1:500) was used for counterstaining the macrophage nuclei. Negative controls consisted of slides in which only the second antibody was added. Slides were examined using radiance 2100-confocal imaging system (Bio-Rad), and visualized by a Green HeNe (543 nm) laser and red laser diode (637 nm) (Nikon E600 microscope is an upright Microscope with Plan Apo 60x/1.4 oil Dic objective).

Binding and cross-linking assays macrophages incubated with recombinant PON1

Binding experiments were carried out essentially as described [22]. Briefly, recombinant PON1 protein was labeled using FITC. Cells were grown in 24-well multidishes and incubated (2 h on ice) with binding buffer (RPMI 1640, 10 mM HEPES, 0.2% BSA) containing increasing concentrations of labeled PON1.

Cells were then washed with ice-cold PBS, solubilized with 200 μl of 1 M NaOH.

For Cross-linking experiments, cells were grown in 150-mm^2 dishes and incubated for 2 h on ice with binding buffer containing 2 mg/ml labeled PON1. The cross linker sulfo-EGS (Pierce; 0.2 mM) was then added for 10 min, followed by quenching with 50 mM Tris–HCl, pH 7.5.

Macrophage plasma membrane preparation

Cells were then washed twice with PBS at 4 °C, scraped and collected and homogenized for 15 s with polytron homogenizer using cold homogenizer buffer)20 mM Tris–HCl, 2 mM EDTA, 0.5 mM EGTA, 5 mM $MgCl_2 \cdot 6H_2O$, 330 mM Sucrose and Trypsin inhibitor) at 4 °C. Then cells were centrifuge for 8 min at 1500 rpm using tablet centrifuge. Supernatant was removed and span at 100,000*g* (50,000 rpm in a Beckman TLA 100.3 rotor with appropriate Beckman polyallomer tubes) for 30 min at 4 °C. Supernatant was poured off and pellet was resuspended and fluorescence was measured by fluorimeter.

Labeling PON1 protein with FITC

Recombinant PON1 protein (2.5 mg/ml) was dialyzed overnight at 4 °C against several changes of borate buffer containing 0.1 M borate, 25 mM sodium tetraborate, 75 mM NaCl, pH 8.6. Prior to conjugation (1 h), the pH of the dialysis buffer was altered to 9.4. Fluorescein isothiocyanate (FITC; Sigma–Aldrich) was dissolved in dimethyl formamide (Merck) and added drop wise to the PON1 solution to give a final concentration of 0.2 mg/ml and then incubated for 1 h at room temperature with stirring. FITC-conjugated PON1 was separated from unconjugated FITC by size exclusion chromatography over a PD-10 column (Amersham-Pharmacia Biotech), eluting with 10 mM phosphate buffer, pH 8.0. FITC-labelled PON1 (2 mg/ml) was used immediately in uptake studies at 0.1–0.8 mg/ml added to J774a1 cell line as describe above.

Macrophages cholesterol efflux

J774 A.1 murine macrophage cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum.

The cells were incubated with mice sera (in order to prevent interactions between antibodies and macrophages Fc receptors) for 30 min, washed and then with [^{3}H]-cholesterol for 1 h at 37 °C. After washing, the cells were further incubated with no addition, either with HDL with or without rePON1 and apoA-I AB's APoA-1 polyclonal IgG, Supplier: Santa Cruz Biotechnology) for 3 h at 37 °C. After cell wash (three times with PBS), 0.1 N NaOH was added for protein determination. Cellular and medium ^{3}[H] labels were quantitated, and the percentage of cholesterol efflux was calculated as the ratio of (^{3}[H] label in the medium) * 100/(^{3}[H]label in the medium + cells) [23]. HDL-mediated cholesterol efflux is the value obtained in the presence of HDL minus the value obtained in cells incubated with no addition of HDL. The extent of cholesterol efflux with no addition of HDL was 4.7 ± 0.3%.

Macrophage-mediated oxidation of LDL

Before oxidation, LDL (1 mg/ml protein) was dialyzed against phosphate-buffered saline. LDL (100 μg/ml protein) in RPMI 1640 medium (without phenol red) was incubated without cells or with J774A.1 cells in the presence of ±2.5 μmol/L $CuSO_4$ for 5 h at 37 °C.

HDL with or without rePON1 and apoA-I AB's (either in free form or bound to rHDL or PC/FC) were added to the incubation medium of LDL with the cells. The extent of cell-mediated oxidation was then determined by the TBARS assay [24].

Statistics

Statistical analysis was performed using Student's paired *t*-test when comparing the mean of two groups. Analysis of variance (ANOVA) was used when more than two groups were compared. Results are given as means ± SD. Each experiment was repeated, separately three times ($n = 3$), and was always performed in triplicates.

Results

PON1 uptake by macrophages

We first analyzed PON1 in macrophages following cell incubation with PON1, by using confocal microscopy analysis. This analysis revealed that whereas in control cells no indication of PON1 protein presence could be shown (Fig. 1A) as expected (since macrophages do not express PON1), PON1 protein was accumulated in the macrophage cytoplasm following cell incubation with PON1 (Fig. 1B). We next questioned whether PON1 cellular uptake is

mediated through PON1 specific binding to the macrophage cell membrane. Macrophages were incubated with labeled PON1 at increasing concentrations following by cell membrane separation. Incubation of macrophages with 0.1, 0.3 or 0.4 mg/ml of PON1 resulted in increasing fluorescence intensity by 17%, 42%, and 62%, respectively (Fig. 1C).

Specificity of PON1 binding to macrophages

We next analyze the specificity of PON1 binding to macrophages by competition assays. Increasing concentrations of FITC-labeled PON1 were added to macrophages in the absence or presence of excess non-labeled PON1 at 4 °C for 2 h. Incubation of cells with FITC-labeled PON1 at concentrations of 0.01, 0.02, 0.04, 0.1 or 0.2 mg/ml increased mean fluorescence intensity by 0%, 14%, 71%, 228% or 242%, respectively (Fig. 2A).Upon adding excess non-labeled PON1 (7.5 mg/ml), mean fluorescence intensity was increased by 0%, 15%, 50%, 128% or 143%, respectively. PON1 binding specificity to macrophages was then calculated by subtraction of labeled PON1 macrophage binding rates from those obtained in the presence of excess non-labeled PON1(Fig. 2A). When similar study was conducted at 37 °C, incubation of macrophages with labeled PON1 at concentrations of 0.1, 0.2 or 0.7 mg/ml increased mean fluorescence intensity by 8-, 12-, and 13-fold, respectively (Fig. 2B). Cell incubation at 37 °C in the presence of excess (7.5 mg/ml) non-labeled PON1, revealed that mean fluorescence intensity was increased by 8-, 11-, and 12-fold, respectively (Fig. 2B).

HDL (but not LDL) binds to macrophage PON1 binding sites

As HDL is the natural carrier of PON1 in serum, we next studied the binding of HDL to macrophages via its PON1 component. As a control we used LDL, which does not contain any PON1. HDL decreased FITC-labeled PON1 binding to macrophages by 30% (Fig. 3A), an effect that was achieved at a concentration of 0.2 mg/ml of PON1. In contrast, upon using LDL as a competitor, no significant effect on PON1 binding to macrophages could be seen along all PON1 concentrations studied (Fig. 3B).

The effect of specific PON1 binding to macrophages on PON1 biological activities

PON1 was previously shown to stimulate HDL-mediated cholesterol efflux from macrophages [25], and to inhibit macrophage-mediated oxidation of LDL [7]. We thus next questioned whether PON1 ability to bind specifically to macrophages is essential for its biological activities. We also compared the contribution of PON1 anti-atherogenicity to that of another HDL-associated protein, apoAI, which is known to possess anti-atherogenic properties [26].

The effects of PON1 and that of apoAI binding to macrophages, on macrophage-mediated LDL oxidation (Fig. 4A) and on HDL-mediated cholesterol efflux from macrophages (Fig. 4B) were analyzed by using PON1 and apoAI antibodies at concentrations that blocked their binding to the cells. Upon adding HDL to macrophages, cell-mediated oxidation of LDL was decreased by 40% in a comparison to control (no HDL added, Fig. 4A). PON1 antibody

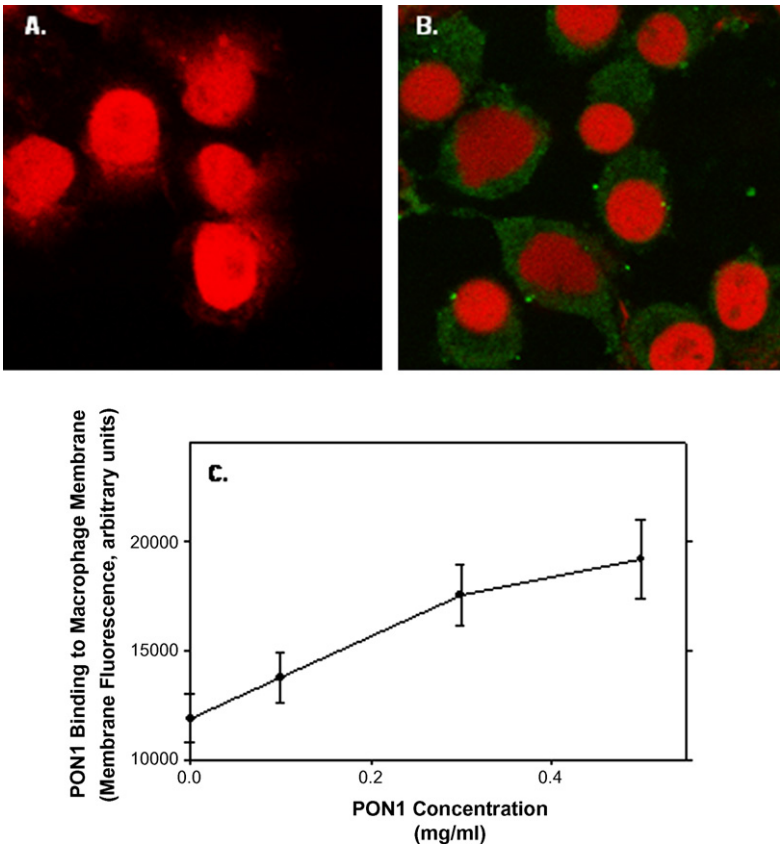

Fig. 1. PON1 association to macrophages cell membrane. Confocal laser scanning microscopic staining view of PON1 in J774A.1 macrophages following cell incubation at 37 °C for 4 h with: (A) no addition, (B) with the addition of recombinant PON1 (0.4 mg/ml). (C) J774A.1 macrophages were incubated with labeled PON1, following by the addition of the cross linker sulfo-EGS (0.2 mM) for 10 min, in order to maintain the interaction between labeled PON1 and macrophages. Macrophage's membranes were separated and PON1 activity was analyzed. Results represent means ± SD of three experiments.

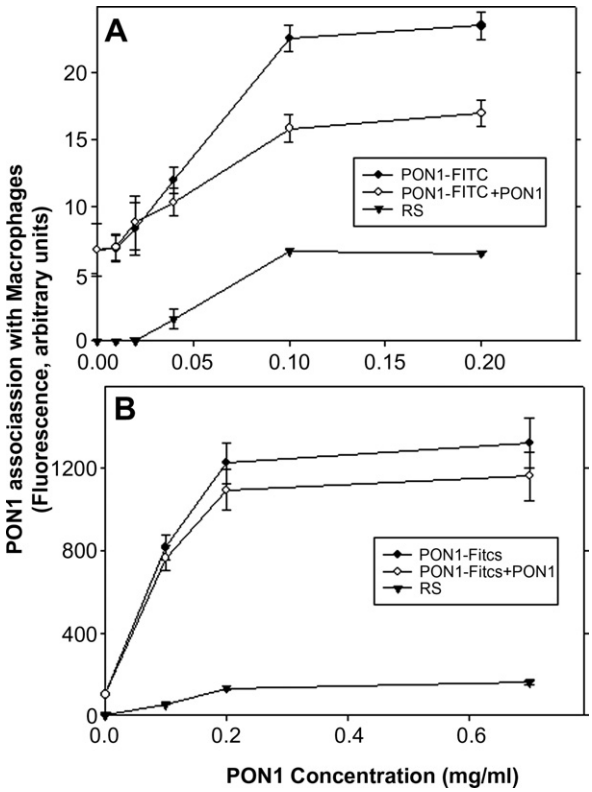

Fig. 2. Specific binding and uptake of recombinant PON1-FITC by macrophages. Recombinant PON1 (rePON1) was labeled with FITC. (A) Using increasing PON1 concentrations (0.1–0.7 mg of protein/ml), PON1s binding to J744A.1 was determined in the absence or presence of excess non-labeled PON1 (7.5 mg/ml) after 2 h at 4 °C. (B) Similarly, analysis was performed at 37 °C. Receptor specificity (RS) was calculated as (PON1 − FITC)−(PON1 FITC + PON1). Results represent means ± SD of two different experiments.

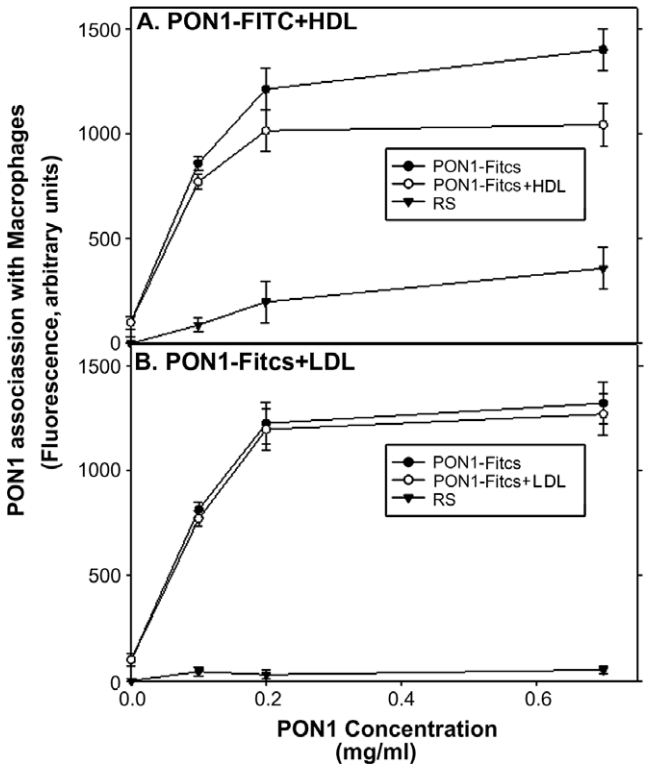

Fig. 3. Specific association of PON1 or HDL, but not LDL, to macrophages. Recombinant PON1 was labeled with FITC. (A) Using increasing PON1 concentrations (0.1–0.7 mg of protein/ml), and incubation time of 4 h at 37 °C PON1s binding to J744A.1 macrophages was determined after incubation with excess, non-labeled HDL (1.5 mg/ml). (B) A similar study as in (A), but incubation with excess of non-labeled LDL. Receptor specificity (RS) was calculated as (PON1 − FITC)−(PON1 FITC + lipoprotein). Results represent means ± SD of two different experiments.

or apoAI antibody decreased macrophages-mediated LDL oxidation by 32% or by 25%, respectively (Fig. 4A). Similarly, HDL increased cholesterol efflux from macrophages by 35%, and upon adding apo-AI or PON1 antibodies to the incubation system,HDL-mediated cholesterol efflux from macrophages was decreased by 35% or by 19%, respectively (Fig. 4B). Finally, in order to exclude the possibility that PON1 antibody directly affected PON1 catalytic activity, recombinant PON1 was incubated without or with PON1 antibody for 2 h. No significant effect on PON1 arylesterase activity was shown (2005 ± 18 U/ml, vs. 2000 ± 15 U/ml, without or with PON1 antibody, respectively).

Discussion

The present study demonstrates, for the first time, that PON1 specifically binds to macrophage plasma membrane via binding sites which are shared by HDL, the carrier of PON1 in serum. Macrophage-bound PON1 is internalized and accumulate in the cytosolic compartment. It was previously shown [9] that when MPM from PON1^0 mice were incubated with human PON1, a 40% increase in PON1 activity was noted. It is of interest that PON1 accumulates in macrophages at the same compartment as PON2 protein does [27], which raises the possibility for an interaction between internalized exogenous PON1 and cellular endogenous PON2, thus allowing for a better protection of macrophages from oxidative stress. PON1 ability to specifically bind to macrophages is necessary for providing PON1 biological activities. PON1 association with HDL involves PON1 N-terminal region and HDL phospholipids [28,29]. PON1 could have been competitively removed from HDL by macrophage membrane phospholipids, suggesting that PON1's retained N-terminal peptide may allows for the transfer of PON1 between phospholipid surfaces. Phospholipids were shown to stimulate and to stabilize PON1 activity [29,30] and it is possible that PON1 binds to the macrophages plasma membrane via cellular phospholipids. PON1 binding to macrophages reached saturation, but with relatively low specificity, suggesting a limited number of receptors for PON1 on macrophages. Both HDL and LDL specifically bind to macrophages [31,32]. HDL, but not LDL however, was able to compete with PON1 for PON1 binding to macrophages, suggesting that HDL component (PON1?) is responsible for HDL competition with labeled PON1 for PON1 binding sites on macrophages.

HDL, but not LDL was also shown to be able to facilitate PON1 secretion from cells [28]. A "desorption" mechanism was proposed, whereby PON1 is inserted into the external membrane of cells from which it may be transferred to HDL during a transient association of the lipoprotein with the cell membrane. Another possible mechanism could be HDL binding via macrophage SR-B1 that anchors HDL to the cell membrane in order to allow for an exchange of PON1 from the lipoprotein to the cell surface. Another option is that PON1 could bind to the macrophage membrane phospholipids and then enters the cell, via a "flip flop" transport mechanism.

PON1 has been demonstrated in atherosclerotic plaque areas, but not in healthy regions of the artery [33], and an active enzymes have been extracted from such plaques [34]. In this respect the biological activities of PON1 which requires its specific interaction with the macrophage binding sites are probably most important.

PON1 protects against macrophage-mediated LDL oxidation [8], and increases HDL binding to macrophages which, in turn, stimu-

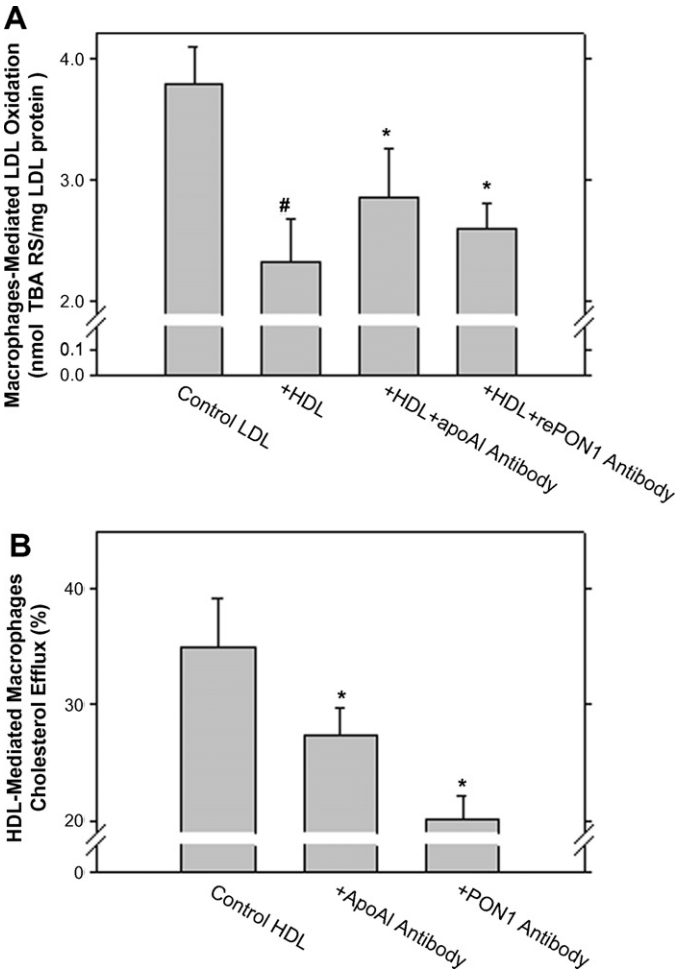

Fig. 4. The effect of PON1 binding to macrophages on PON1 biological activities: HDL was pre incubated with either PON1 or with apoAI antibodies for 2 h at 37 °C. The HDL samples (100 μg protein/ml) were then further incubated with J774A.1 macrophages prior to biological activities assays. (A) macrophages-mediated oxidation of LDL (100 μg protein/ml) was determined by the TBARS assay after 6 h of incubation at 37 °C in the presence of 5 μmol/L of $CuSO_4$. (B) The HDL fractions were added to macrophages that were pre-labeled with 3[H]-cholesterol. The extent of HDL-mediated cholesterol efflux from macrophages was determined as described under the Methods section. Results represent means ± SD of two different experiments. $^{\#}p < 0.01$ vs. no HDL, $^{*}p < 0.01$ vs. control HDL.

lates HDL ability to promote cholesterol efflux. These two major anti-atherogenic properties of HDL (and of PON1), requires, at least in part, macrophages binding sites for HDL-associated PON1.

In conclusion then, PON1, as well as HDL-associated PON1, specifically binds to macrophage, leading to anti-atherogenic effects. Macrophage PON1 binding sites may thus be a target for future cardio protection therapy.

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