Reactive Dicarbonyl Scavenging Effectively Reduces MPO-Mediated Oxidation of HDL and Preserves HDL Atheroprotective Functions

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Abstract:

High-density lipoprotein (HDL) is atheroprotective by mediating cholesterol efflux, anti-inflammatory, and anti-oxidation functions. Atheroprotective functions of HDL are related to the activity of HDL-associated enzymes such as paraoxonase 1 (PON1). We examined the impact of inhibition of myeloperoxidase (MPO)-mediated HDL oxidation by PON1 on HDL malondialdehyde (MDA) content and HDL function. In the presence of PON1, crosslinking of apoAI in response to MPO-mediated oxidation of HDL was abolished and MDA-HDL adduct levels were decreased. In addition, PON1 prevented the impaired cholesterol efflux capacity of MPO-oxidized HDL from *Apoe*$^{-/-}$ macrophages. Direct modification of HDL with MDA increased apoAI crosslinking and reduced the cholesterol efflux capacity in a dose dependent manner. In addition, MDA modification of HDL reduced its anti-inflammatory function compared to native HDL as the expression of IL-1β and IL6 increased by 3- ($p<0.05$) and 1.8-fold ($p<0.05$) in *Apoe*$^{-/-}$ macrophages in response to LPS. MDA-HDL also had impaired ability to increase PON1 activity. Importantly, HDL from subjects with familial hypercholesterolemia (FH-HDL) versus controls had increased MDA-apoAI adducts, and normalization of the PON1 activity to PON1 mass revealed a 24 % ($p<0.05$) decrease in specific activity indicating that PON1 activity is also impaired in FH. Consistent with the impaired PON1 activity and increased MDA-apoAI, FH-HDL induced a pro-inflammatory response in *Apoe*$^{-/-}$ macrophages compared to incubation with LPS alone. FH-HDL versus control HDL also had an impaired ability to promote cholesterol efflux from *Apoe*$^{-/-}$ macrophages. Interestingly, reactive dicarbonyl scavengers effectively abolished MPO-mediated apoAI crosslinking, MDA adduct formation, and improved cholesterol efflux capacity. Importantly, in vivo treatment of hypercholesterolemic mice with reactive dicarbonyl scavengers effectively reduced MDA-HDL adduct formation and increased PON1 activity and HDL cholesterol efflux capacity, supporting a therapeutic potential of reactive carbonyl scavenging in maintaining HDL function.

Keywords: Familial Hypercholesterolemia, Cholesterol efflux, Macrophages, Malondialdehyde (MDA), Reactive Dicarbonyl Scavengers, High-density lipoprotein (HDL)

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disorder, most commonly due to mutations in the genes for *LDLR*, *ApoB* and *PCSK9*, characterized by remarkably increased levels of LDL-C and high risk of premature coronary artery disease [1]. The high risk of premature cardiovascular disease (CVD) is attributable to the increased levels of LDL-C and oxidized LDL, with little attention paid to the role of HDL function. The relationship between HDL-C levels and risk for atherothrombosis is complex, as there are apparent exceptions to the inverse relationship at both ends of the HDL-C spectrum [2]. Furthermore, recent evidence suggests that HDL particle number may be a better gauge than HDL-C levels for CVD protection. Recent studies have shown that HDL cholesterol efflux capacity is negatively associated with carotid and coronary atherosclerosis and atherosclerotic cardiovascular events independent of HDL-C levels [3-6].

Myeloperoxidase (MPO) is a heme-containing enzyme released from azurophilic granules of polymorphonuclear neutrophils and monocytes into the extracellular fluid in the setting of inflammation. The MPO-derived hypochlorous acid (HOCl) oxidizes HDL in human atherosclerotic lesions and reduces ABCA1-dependent cholesterol efflux by site-specific chlorination of apolipoprotein A-I (apoAI) tyrosine residues [7-10] . In addition, modification of HDL by MPO generates a pro-inflammatory particle [11, 12]. These findings suggest that MPO peroxidase activity promotes the formation of dysfunctional HDL in human atherosclerosis. Besides direct oxidation of apoAI, MPO derived oxidants also modify lipids generating highly reactive dicarbonyls such as malondialdehyde (MDA) and isolevuglandins (IsoLGs) which covalently bind apoAI and reduce cholesterol efflux (Fig. 1) [13, 14]. Therefore, we examined the ability of the dicarbonyl scavengers, 2-

hydroxylbenzylamine (2-HOBA) and pentyl-pyridoxamine (PPM), to modulate HDL function by neutralizing reactive dicarbonyls (Fig. 1).

The chronic inflammatory atherosclerotic process has been associated with reduced activity of the HDL-associated anti-oxidative enzyme, paraoxonase 1 (PON1) [15]. PON1 is associated with increased plasma HDL levels [16]. While the in vivo substrates of PON1 remain to be identified, PON1 activity (Fig. 1) prevents the accumulation of lipid hydroperoxides in HDL and LDL[17]. PON1 also inhibits N-homocysteinylation of LDL-associated proteins by hydrolyzing the highly reactive pro-oxidant homocysteine thiolactone [18]. In addition, PON1 activity neutralizes bioactive oxidized 1-palmitoyl-2-arachidonoyl phosphatidylcholine [19]. Recent studies found that apoAI forms a functional ternary complex with PON1 and MPO [20]. However, the impact of inhibition of MPO-mediated HDL oxidation by PON1 on HDL function is still unknown. We examined the impact of HDL-associated PON1 in preventing MPO-mediated HDL MDA modification and dysfunction, and the potential role of reduced PON1 activity in promoting increased MDA modification and impaired function of HDL from FH patients. Here, our findings demonstrate that PON1 inhibition of MPO activity prevents MDA-HDL adduct formation and crosslinking of apoAI and preserves the cholesterol efflux capacity of HDL. In addition, direct modification of native HDL with MDA increases apoAI crosslinking and impairs the ability of HDL to enhance PON1 activity, reduce inflammation, and mediate cholesterol efflux. Interestingly, subjects with FH have decreased PON1 activity, and FH-HDL contains cross-linked apoAI and MDA-apoAI adducts that likely contribute to the impaired cholesterol efflux and anti-inflammatory functions of FH-HDL. Importantly, administration of reactive dicarbonyl scavengers to hypercholesterolemic mice increased PON1 activity and HDL cholesterol efflux capacity demonstrating the therapeutic potential of reactive dicarbonyl scavengers in preserving HDL atheroprotective functions.

Materials and Methods

Reagents — Human neutrophil myeloperoxidase (MPO) was obtained from Lee Biosolutions Inc. (St. Louis, MO). Human paraoxonase1 (PON1) recombinant protein was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Human apolipoprotein A-I (apoAI), and dimethyl sulphoxide (DMSO) were obtained from Alfa Aesar (Ward Hill, MA). Fresh HDL was purified from FH patients and normal subjects following the HDL purification kit's instructions (Cell Biolabs Inc., California). Maloncarbonyl bis-(dimethylacetal), taurine and H_2O_2 were purchased from Sigma-Aldrich (St. Louis, MO). PON1 ELISA kit and EnzCheck PON1 activity kit were purchased from R&D systems Inc. (Minneapolis, MN) and Invitrogen (Carlsbad, CA), respectively. MDA-HDL ELISA kit was purchased from Cell Biolabs Inc. (San Diego, California). The chemiluminescent Western Lightning ultra-reagent was obtained from PerkinElmer Inc. (Waltham, MA). L-012 was obtained from Wako Chemicals (Richmond, VA). Primary antibody to apoAI was purchased from Meridian Life science (Memphis, TN) and the secondary antibody HRP-conjugated anti-goat IgG from rabbit was purchased from Sigma-Aldrich (St. Louis, MO).

Working solutions of H_2O_2 were made fresh daily by diluting 30% H_2O_2 (BDH Chemicals, London, UK) according to the extinction coefficient for H_2O_2 at 240 nm, 39.4 $M^{-1}cm^{-1}$ [21]. Buffers were used in L-012 luminescence assay, including PS6.5 buffer (130mM NaCl, 6.28mM Na_2HPO_4, 18.7mM NaH_2PO_4), and MAPS imaging solution (buffer Cit6 with 20mM NaBr, 200mM $(NH_4)_2SO_4$, 100 p.p.m. v/v Tween20, 50 μM L-012 and 100 μM (H_2O_2), 1ppm = 0.0001%)) [22, 23].

Mice — Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in microisolator cages with *ad libitum* access to a rodent chow diet containing 4.55 % fat (PMI 5010, St. Louis, Mo) and water. The mouse protocols were

approved by the Institutional Animal Care and Use committee of Vanderbilt University. Experimental procedures and animal care were performed according to the regulations of Vanderbilt University's Institutional Animal Care and Usage Committee.

Reactive Dicarbonyl Scavengers—We used 2-HOBA and PPM to examine the effects of reactive dicarbonyl scavenging on HDL function in vitro and in vivo. In some experiments, taurine was used as a scavenger. PPM, 2-HOBA, and taurine were easily solubilized in water and prepared as a 100 mM stock and stored as small aliquots in -80ºC until use. Fresh working solutions were prepared before each assay and diluted in water to appropriate concentrations. For in vivo studies, after pretreatment of mice on a chow diet with 2-HOBA for 2 weeks, 7 week old female $Ldlr^{-/-}$ mice on a western diet were treated with 1 g/L of 2-HOBA for either 8 weeks or 16 weeks.

Human Blood Collection, ApoB-depleted Serum Preparation, and Lipoprotein Isolation—The study was approved by the Vanderbilt University Institutional Review Board (IRB), and all participants gave their written informed consent. The human blood from FH patients and healthy controls were obtained using an IRB approved protocol. ApoB-depleted serum was prepared as described [24]. Briefly, to remove apoB-containing lipoproteins from serum of fasted subjects, 400 µL of PEG 8,000 (Sigma-Aldrich, St. Louis, MO] (20% in 200 mM glycine) was added to 1 ml serum, incubated for 15 min at room temperature, and then centrifuged at 1,900g for 20 min. The supernatant containing the apoB-depleted serum was collected and used. HDL and LDL were prepared from serum by Lipoprotein Purification Kits (Cell BioLabs, Inc.).

Peritoneal Macrophages — Peritoneal macrophages were isolated from 3 to 4 month old *Apoe*$^{-/-}$ mice (Jackson Laboratories, Sacramento, CA). Four days after injection of 10% of

thioglycolate, cells were obtained by peritoneal lavage with ice-cold Ca^{2-} and Mg^{2-} free PBS. Peritoneal cells were centrifuged and re-suspended in DMEM supplemented with 10% heat-inactivated FBS and 100 units/ml penicillin/streptomycin. Cells were plated onto 24-well culture plates ($1*10^6$ cells/well) and allowed to adhere for 2 h. Non-adherent cells were removed by washing two times with DPBS, and adherent macrophages were used for experiments.

Modification of apoAI and HDL with MDA — MDA was prepared immediately before use by rapid acid hydrolysis of maloncarbonyl bis-(dimethylacetal) as described [13]. Briefly, 20 µL of 1 M HCl was added to 200 µL of maloncarbonyl bis-(dimethylacetal), and the mixture was incubated for 45 min at room temperature. The MDA concentration was determined by absorbance at 245 nm, using the coefficient factor 13, 700 M^{-1} cm^{-1}. ApoAI (1mg protein / mL) or HDL (10mg of protein /mL) and increasing doses of MDA (0, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM) were incubated at 37 ^{o}C for 24 h in 50 mM sodium phosphate buffer (pH7.4) containing DTPA 100 µM. Reactions were initiated by adding MDA and stopped by dialysis of samples against PBS at 4 ^{o}C.

*Modification of HDL with MP*O — The MPO-mediated HDL modification reactions were performed in 50 mM phosphate buffer (pH 7.0) containing 100 µM DTPA, 1 mg/ml protein of HDL, 70 nM purified human MPO (A430/A280 ratio of 0.79), and 100 mM chloride [25]. For the PON1 inhibition reaction, 1 U/mL PON1 was incubated with 1mg/mL HDL for 10 min and then 70 nM MPO, 160 uM H_2O_2, and 100 mM NaCl were added to initiate the reaction. The reactions were carried out at 37 °C for 1 h. In all reactions, the concentration of the key reactants was verified spectrophotometrically using molar extinction coefficients of 170 cm^{-1} mM^{-1} at 430 nm for MPO and 39.4 cm^{-1} mM^{-1} at 240 nm for H_2O_2.

MDA-apoAI and MDA-HDL adduct ELISA — The ELISA assays for MDA adduct levels were performed essentially following the manufacturers instructions (Cell Biolabs, Inc.) instructions. Briefly, 96-well coated plates were blocked by adding 100 μL of blocking buffer (1% BSA, 0.05% NaN3, in PBS, pH 7.2-7.4) and incubated at room temperature for 2 h. The diluted samples and standard curve samples (5 ng/ml) were added to wells and incubated for 2 h at room temperature with shaking at 600-800 rpm. After washing, 100 μL of detection antibody was added to the wells and incubated for 2 h. Then HRP-Streptavidin was added and incubated for 30min at room temperature. 100 μL of substrate was added and incubated at room temperature for 5 min. After stopping the reaction, the OD values were determined at 450nm.

PON1 ELISA —The PON1 levels were measured using the ELISA assay (R&D systems, Inc.) following the manufacturer's instructions with a few modifications. Briefly, unknown serum samples (diluted 1:500) and the PON1 standards were added to wells coated with the PON1 capture antibody and incubated for 2 h. Each well was then rinsed 5 times with 200 μl of washing buffer, and 100 μl of blocking solution was added to each well. After incubation for 2h at room temperature, the biotinylated goat anti-human PON1 antibody (diluted 1:1000) was added to detect the captured PON1 from serum and incubated for 2 h. The wells were then washed 5 times, incubated for 1 h with streptavidin-HRP conjugate, then incubated with substrate solution for 20 min at room temperature. After stopping the reaction, the OD was measured at 450nm wavelength.

Analysis of PON1 Activity— The PON1 activity assay was performed using the EnzCheck PON1 activity kit (Thermo Fisher Scientific), which measures the organophosphatase activity of PON1. PON1 standards and diluted human serum samples were prepared in TSC buffer (20mM Tris-HCl, 150mM NaCl and 2mM $CaCl_2$, pH 8.0). The samples and

standards were then incubated for 30 min with TSC buffer containing 100μM substrate. Fluorescence intensity was then measured using Biotek's Synergy MX Microplate reader (Winooski, VT) with Gen 5 software at excitation wavelength of 368nm and an emission wavelength of 460nm after 30 min of incubation at 37 ^{0}C.

MPO Peroxidase Activity Assay —The MPO luminescence assay was performed as described [26]. To evaluate the inhibitory effect of PON1 on MPO activity, a luminescence assay was developed with L-012 as the peroxidase substrate. L-012 oxidation was monitored as a function of luminescence intensity over time using Biotek's Synergy MX Microplate reader (Winooski, VT) with Gen 5 software. PON1 (0, 0.12, 0.24, 0.48, 0.94, 1.87, 3.75, 7.5 uM) was incubated with 0.05 mg/mL MPO for 30 min at room temperature, then the samples were loaded into microtiter plate wells and then freshly prepared MAPS imaging solution containing L-012 and H_2O_2 was added. Each well received 75 μL imaging solution and the plate was briefly shaken up to 1,000 rpm and imaged immediately. Data were quantitated at the 5-min time-point.

HDL Cholesterol Efflux Capacity Assay—Cholesterol efflux was measured as previously described [27, 28]. Briefly, *Apoe*$^{-/-}$ macrophages were isolated and incubated for 48 h with DMEM containing acetylated LDL (40 μg/ml) and 1.0 μCi/mL ^{3}H-cholesterol (PerkinElmer, Boston, MA). The cells were then washed and incubated for 24 h in DMEM supplemented with HEPES in the presence of HDL or MDA-HDL. After filtering aliquots of media through 0.45μM multiscreen filtration plates to remove floating cells, the [^{3}H]cholesterol was measured by liquid scintillation counting. Cellular [^{3}H]cholesterol was extracted by incubating the monolayers overnight in isopropanol. Cellular cholesterol content and proteins were measured as described [13, 14].

Measurement of HDL-apoAI exchange (HAE) — HDL at a concentration of 1 mg/mL (total protein) was mixed with 3 mg/mL spin-labeled, lipid-free apoAI in a 3:1 ratio [29, 30]. Samples were drawn into borosilicate capillary tubes (VWR) and incubated for 15 min at 37˚C. Electron paramagnetic resonance (EPR) measurements were performed in a Bruker EMX Nano spectrometer outfitted with a temperature controller set to 37˚C. The peak amplitude of the nitroxide signal (3462-3470 Gauss) was compared with the peak amplitude of a proprietary internal standard (3507-3515 Gauss) provided by Bruker. The internal standard is contained within the instrument and does not contact the sample. Because the *y* axis of the EPR spectrometer is measured in arbitrary units, measuring the sample against a fixed internal standard facilitates normalization of the response. The HDL-apoAI exchange (HAE) activity represents the sample/internal standard signal ratio at 37˚C. The maximal percentage of HAE activity was calculated by comparing HAE activity with a standard curve ranging in the degree of spin-labelel lipid-associated apoA-I signal. Experiments were repeated in duplicate and averaged.

Measurement of free lysine using o-phthalaldehyde (OPA) — OPA is a primary amine-reactive fluorescent detection reagent that is used to detect free lysine in HDL [14, 31, 32]. The procedure was performed according to the manufacturer's instructions (Thermo Scientific) using HDL modified by lipid aldehydes as described above and adapted to 96-well plates. % lysine adduction was calculated as fluorescence of modified HDL/unmodified HDL.

Western Blot for apoAI crosslinking — Protein samples were incubated with reducing reagent β-mercaptoethanol and SDS-PAGE sample loading buffer for 10 min at 55 ^{0}C and then resolved by NuPAGE Bis-Tris electrophoresis. The gels were transferred onto PVDF membranes (Amersham Bioscience) at 150 V for 1.5 h. Blots were blocked with 5% milk

at room temperature for 2 h and probed with primary antibodies specific to human apoAI from goat overnight at 4 °C. The secondary antibodies conjugated with HRP were incubated with the membranes for 2 h at room temperature. Protein bands were visualized with ECL western blotting detection reagents (GE Healthcare).

RNA isolation and Quantitative RT-PCR— Apoe$^{-/-}$ macrophages were incubated for 4 h with 25 ng/mL LPS in the absence or presence of 50 μg/mL of HDL or MDA-HDL (molar ratios of MDA to HDL is 0/1, 5/1, 10/1, 20/1, 40/1). Total RNA was extracted from peritoneal macrophages using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and first-strand cDNA was synthesized from the total RNA (250 ng) using a Reverse Transcription Reagent (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with a Perkin–Elmer 7900 PCR machine, TaqMan PCR master mix and FAM-labeled TaqMan probes (Assays-on-Demand, Applied Biosystems) for IL-6, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples were run in 20 μL reactions using an ABI 7800 (Applied Biosystems). Samples were incubated at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 30 s. Expression data were normalized to GAPDH levels.

Statistical analysis

Results are provided as mean ± the standard error of the mean (SEM). Data were analyzed by Student's t test or one-way ANOVA coupled with Bonferroni-Dunn post hoc test using Prism 6. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of PON1 on MPO activity, apoAI crosslinking, MDA-HDL adducts, and HDL cholesterol efflux capacity.

The MPO peroxidase activity was maximally inhibited by increasing doses (IC50 = 40 nM) of PON1 (Fig 2A), which is consistent with previous studies [20]. In addition, Addition of MPO and its substrates to HDL increased apoAI crosslinking (Fig 2B) [8]. However, this MPO-mediated crosslinking of apoAI in HDL was almost abolished in the presence of 100 nM PON1(Fig 2B). MPO also increased the production of MDA-HDL adducts by 7-fold ($p<0.001$) compared to the MDA content of HDL not treated with MPO (Fig 2C). Importantly, the addition of PON1 reduced MPO-mediated MDA-HDL adduct production by 86% ($p<0.001$) (Fig 2C). Consistent with the increased apoAI crosslinking and MDA-HDL adducts, MPO-mediated oxidation of HDL reduced its cholesterol efflux capacity by 56% (Fig 2D). The inhibition of MPO activity by PON1 prevented the impairment in the cholesterol efflux capacity of HDL (Fig 2D). Taken together, these results suggest that PON1 preserves HDL cholesterol efflux function by inhibiting MPO-mediated apoAI crosslinking and MDA-HDL adduct formation.

Effects of MDA modification of apoAI or HDL on apoAI crosslinking, cholesterol efflux capacity, and HDL-apoAI exchangeability.

As our data demonstrate that PON1 inhibits MPO-mediated apoAI crosslinking and MDA-HDL adduct production, we next examined the effects of direct modification of apoAI or HDL with MDA on apoAI crosslinking and cholesterol efflux. ApoAI (molar ratios of MDA to apoAI: 25/1, 50/1, 100/1) and HDL (molar ratios of MDA to apoAI: 5/1, 10/1, 20/1, 40/1) were directly modified with increasing doses of MDA. Direct modification of apoAI (Fig 3A) and HDL (Fig 3B) with MDA caused crosslinking of apoAI in a dose dependent manner. In addition, direct MDA modification of apoAI (Fig 3C) and HDL (Fig 3D) led to similar MDA

adduct content as was observed with MPO-mediated oxidation of HDL (Fig 2C). Modification with MDA reduced the cholesterol efflux capacity of apoAI (Fig 4A) by 21.8% ($p<0.05$), 22% ($p<0.05$), 55% ($p<0.001$), for apoAI modified with the increasing MDA concentrations (molar ratio of MDA to apoAI of 25/1, 50/1, 100/1, respectively), which is consistent with other studies[13]. In addition, modification with MDA reduced the cholesterol efflux capacity of HDL (Fig 4 B) by ~ 37% ($p < 0.05$), 42% ($p < 0.05$), 62.8% ($p < 0.01$), and 80% ($p < 0.001$), respectively, for 1mg/mL HDL modified with 0.125 mM, 0.25 mM, 0.5 mM, and 1mM MDA (molar ratios of MDA/HDL equivalent to apoAI: 0/1, 1/1 5/1, 20/1, 40/1). We determined the ability of MDA to modify lysine residues on HDL using o-phthalaldehyde (OPA) to detect available lysines. OPA also detects the headgroups of phosphatidylethanolamines (PEs), although these are in much lower abundance than lysyl residues. Modification with MDA (40 eq) reduced the available lysines of HDL by approximately 40% (Figure 4C). As some of the cholesterol efflux capacity of HDL is from dissociated apoAI interacting with ABCA1 [33], we also performed EPR to measure the ability of HDL-apoAI to exchange [29]. Interestingly, modification with 0.025 mM, 0.125mM, 0.25 mM, 0.5 mM, and 1 mM MDA reduced the HDL-apoAI exchangeability by 32%, 69%, 64%, 67%, and 71%, respectively (Fig. 4D). Taken together, these data indicate that MDA modification of HDL increases HDL-apoAI crosslinking resulting in impaired cholesterol efflux and reduced HDL-apoAI exchangeability.

Effects of reactive dicarbonyl scavenging on HDL-apoAI crosslinking and cholesterol efflux capacity during MDA modification and MPO-mediated oxidation. We first determined if the reactive dicarbonyl scavenger, taurine, could prevent the crosslinking of apoAI in HDL directly modified by MDA. Treatment of HDL with 5 mM taurine before addition of MDA (0.25 mM MDA) resulted in a 45% ($p<0.05$) reduction in apoAI crosslinking (Fig 5A). Similar results were seen when apoAI was pretreated with

taurine and modified with MDA (data not shown). Consistent with the reactive dicarbonyl scavenging reducing HDL-apoAI crosslinking, the cholesterol efflux capacity of taurine treated HDL was preserved (Fig 5B). Importantly, reactive dicarbonyl scavenging with 5mM taurine, 0.5 mM 2-HOBA, or 0.5 mM PPM markedly reduced MPO-mediated HDL apoAI crosslinking (Fig 5C-5E). In addition, 0.5 mM 2-HOBA prevented the MPO mediated HDL cholesterol efflux dysfunction (Fig 5F).

Effects of HDL MDA modification and dicarbonyl scavenging on PON1 activity

It has been shown that PON1 interacts with apoAI of HDL resulting in increased enzymatic activity [34]. Native HDL increased the activity of recombinant PON1 by 56% (Fig 6A), whereas MDA modification of HDL reduced its ability to activate PON1 with the highest dose of MDA only increasing activity by 12% (molar ratio of MDA to HDL: 40/1). As administration of an atherogenic diet to *Ldlr*$^{-/-}$ mice increases apoAI oxidation and crosslinking and reduces PON1 activity [35], we examined whether scavenging reactive dicarbonyls improves PON1 activity and HDL cholesterol efflux capacity in *Ldlr*$^{-/-}$ mice consuming a western diet. Treatment of *Ldlr*$^{-/-}$ mice with 1g/L 2-HOBA improved the PON1 activity by 18.5 % (Fig 6B). In addition, treatment of the *Ldlr*$^{-/-}$ mice with the reactive dicarbonyl scavenger, PPM, increased the cholesterol efflux capacity of their HDL by 37.5% (Fig 6C). Consistent with the improved PON1 activity and HDL cholesterol efflux function, the HDL MDA content was reduced by 61% (Fig 6D). In addition, the MDA content of LDL decreased by 57% (Fig 6E).

FH patients have decreased PON1 activity, increased MDA-apoAI adducts, defective HDL cholesterol efflux capacity and impaired HDL anti-inflammatory function.

As hypercholesterolemia impacts PON1 activity in mice, we next examined the PON1 levels and activity in FH patients versus control subjects. After precipitation of the apoB

containing lipoproteins from serum with PEG, PON1 mass and activity were measured in the HDL containing fraction. Compared to control subjects, PON1 levels and activity were decreased by 23 % ($p<0.05$) and 41.7 % ($p<0.05$) in in the FH-HDL fraction, respectively (Fig 7A and 7B). The PON1 activity and levels were not significantly different in the FH-HDL fractions (Fig 7A and 7B) of serum collected pre versus post LDL apheresis (LA). Normalization of the PON1 activity to PON1 mass revealed a 24 % decrease in specific activity indicating that PON1 activity is impaired (Fig 7C). In addition, the levels of MDA-apoAI adducts were increased nearly 3-fold in FH versus control HDL (Fig 7D). In agreement with the increased MDA adducts, the cholesterol efflux capacity of FH-HDL was markedly reduced compared to control HDL (Fig 7E).

To determine whether MDA adducts contributed to the pro-inflammatory status of FH-HDL, the effects of direct MDA modification of HDL on the response to LPS in *Apoe*$^{-/-}$ macrophages was examined. Whereas control HDL inhibited the LPS-induced expression of IL-6 and IL-1β by 60% ($p<0.05$) and 40% ($p<0.05$) (Fig. 8A & 8B), respectively, MDA-HDL (molar ratio of MDA to HDL of 40/1) enhanced the expression of IL-1β and IL-6 by 3-fold ($p<0.05$) and 1.8-fold ($p<0.05$). In addition, MDA reduced the anti-inflammatory function of HDL in a dose dependent manner (Fig. 8A & 8B). As reduced PON1 inhibition of MPO-mediated HDL oxidation produces pro-inflammatory particles[11], we next examined the effectiveness of FH-HDL versus control HDL in preventing the inflammatory response to LPS in *Apoe*$^{-/-}$ macrophages. Unlike control HDL, which reduced the expression of IL-1β and TNF-α in response to LPS, FH-HDL from subjects prior to LA increased the mRNA levels of IL-1β and TNF-α by 400-fold and 10-fold compared to incubation with LPS alone, respectively (Fig 8C and 8D), which indicates that FH-HDL is extremely pro-inflammatory. Similarly, FH-HDL post LA induced a pro-inflammatory

response to LPS (Fig 8C and 8D). Thus, it is possible that MDA-HDL adducts contribute to the inflammatory nature of both MPO oxidized HDL and FH-HDL.

DISCUSSION

Oxidative modifications of HDL proteins and lipids result in compromised function, and evidence has mounted that HDL dysfunction is atherogenic. The current studies show that HDL-associated PON1 is effective at preventing MPO-mediated HDL modification and apoAI crosslinking, which preserves HDL anti-inflammatory and cholesterol efflux functions. In addition, we show that MDA modification of HDL results in reduced ability to activate PON1 which may be relevant to FH patients who have increased plasma MDA-HDL adducts that likely contribute to their decreased PON1 activity and HDL dysfunction. In vitro inhibition of MPO generated reactive dicarbonyls with the scavengers, PPM and 2-HOBA, prevented HDL apoAI crosslinking and cholesterol efflux dysfunction. Importantly, in vivo scavenging of reactive dicarbonyls improved PON1 activity and HDL cholesterol efflux function supporting this as a potential therapeutic strategy for reducing atherosclerosis.

MPO mediates the chlorination, nitration, and direct oxidation of proteins as well as initiating lipid peroxidation causing the generation of highly reactive dicarbonyls (Fig 1). Plasma MPO levels predict risk of clinical events in subjects with CVD, and human atherosclerotic lesions are markedly enriched in MPO activity [36-38]. MPO interacts directly with apoAI, and this interaction is enhanced with MPO oxidized apoAI [39] causing accumulation of apoAI that is extensively crosslinked and dysfunctional within atherosclerotic lesions. Recent studies showed that PON1 forms a ternary complex with apoAI and MPO, and that PON1 interaction with apoAI decreases MPO activity [20]. In addition, interaction of PON1 with apoAI activates the enzyme and enhances its ability to

hydrolyze oxidized phospholipids. The current studies show that the addition of PON1 to HDL and MPO essentially abolishes MPO-mediated apoAI crosslinking and MDA adduct content, thereby effectively maintaining HDL cholesterol efflux capacity (Fig. 2). Interestingly, we show that MDA modification of HDL reduced the ability of HDL to activate PON1 (Figure 6A), raising the possibility that MDA adduct formation interferes with apoAI activation of PON1. In this regard, a prior study showed that MDA targets the K_{206} and K_{208} residues of lipid-free apoAI to form Lysine-MDA-Lysine adducts [13], and studies by Huang and colleagues[20] demonstrated that the peptide region, S_{201}TLSEKAK_{208}, is critical to PON1 interaction with apoAI. Indeed, mutations in this region (S_{201}TLSEKAK_{208} to S_{201}ALAAEAE_{208}) designed to decrease ionic interactions markedly reduce PON1 interaction and activation resulting in an impaired ability to decrease MPO activity [20]. Taken together, it is likely that modification of apoAI with Lysine-MDA-Lysine adducts impairs PON1 interaction with HDL. Consistent with this possibility, treatment of *Ldlr*$^{-/-}$ mice with reactive dicarbonyl scavengers significantly increased their PON1 activity and decreased MDA-HDL adducts (Figure 6).

The current studies demonstrate that MDA-HDL adducts are a major product of MPO mediated oxidation of HDL (Fig 2). Direct MDA modification of lipid-free apoAI caused intermolecular crosslinking (Fig 3), and, consistent with other studies, MDA-apoAI adduct formation decreased cholesterol efflux in a MDA dose-dependent manner [13]. Similar to MDA-apoAI, MDA modification of HDL caused extensive apoAI crosslinking. In addition, MDA modification of HDL likely generated apoAI/apoAII heterodimers as evidenced by the presence of a 38kDa band which was absent with MDA-apoAI (Fig 3). Similar to MDA-apoAI, MDA modification of HDL impaired its cholesterol efflux capacity (Fig 4). MDA-HDL also had a reduced ability to prevent the macrophage inflammatory response to LPS (Fig 8). MDA-HDL had decreased apoAI exchangeability, which likely contributes to the

impaired cholesterol efflux and anti-inflammatory functions. Studies have shown that a significant portion of cholesterol efflux to HDL occurs via ABCA1, which releases cholesterol and phospholipids to ApoAI, some of which has dissociated from HDL particles [40, 41]. Furthermore, exchangeable apoAI can reduce the inflammatory response to LPS via ABCA1 mediated activation of STAT3 [42]. Consistent with this concept, HDL function via ABCA1 is correlated with HDL apoAI exchangeability [27]. Studies have also shown that decreased HDL apoAI exchangeability is associated with the presence of atherosclerosis in humans raising the possibility that MPO-mediated MDA modification of HDL impacts atherosclerosis development [27].

Our studies show that FH-HDL versus control HDL contained 3-fold more MDA-Lysine adducts. Compared to control HDL, FH-HDL had impaired cholesterol efflux and anti-inflammatory function (Fig 8). In addition, FH patients had decreased PON1 activity compared to controls (Fig 7). The increased MDA content of FH-HDL probably contributes to its dysfunction as direct MDA modification of control HDL impaired PON1 activity, cholesterol efflux potential, and anti-inflammatory function. However, it is likely that other factors contribute to the dysfunction of FH-HDL, especially given the extremely proinflammatory effects of the particles. A number of apoAI oxidative modifications have been shown to decrease the anti-inflammatory function of HDL including chlorinated tyrosine and oxidized tryptophan 72 [12, 31, 43]. Furthermore, our recent studies showed that FH versus control HDL contain increased isolevuglandins which are highly reactive γ-ketoaldehydes [14], and direct modification of HDL with isolevuglandins caused a proinflammatory response to LPS. In addition to oxidation products, other factors such as LCAT, CETP, microRNAs, apoL1, and sphingosine-1-phosphate could impact the anti-inflammatory function of FH-HDL [44].

Our studies show that the lowering of LDL-C with LDL apheresis has little impact on FH-HDL dysfunction and recent studies demonstrated that HDL function is an independent predictor of CAD. These findings highlight that in addition to cholesterol lowering drugs, therapeutic treatments which preserve HDL function are needed to further reduce CAD risk. The current studies show that the reactive dicarbonyl scavenger, 2-HOBA, eliminated MPO mediated HDL apoAI crosslinking, MDA adducts, and cholesterol efflux dysfunction (Fig 5). Importantly, in vivo administration of reactive dicarbonyl scavengers to *Ldlr*$^{-/-}$ mice consuming a western diet, which increases oxidative stress, enhanced PON1 activity and HDL cholesterol efflux capacity (Fig 6). Consistent with the improved HDL function, in vivo treatment with reactive dicarbonyl scavengers also reduced the MDA-HDL adducts. Taken together, our studies suggest that reactive dicarbonyl scavengers have therapeutic potential in preventing HDL dysfunction. In addition to preventing MDA-HDL adduct formation, in vivo treatment of *Ldlr*$^{-/-}$ mice with PPM markedly reduced MDA-LDL adduct formation (Fig 6). Thus, in vivo reactive dicarbonyl scavenging is also atheroprotective by preventing reactive aldehyde modification of LDL, which causes enhanced uptake by macrophages and accelerated foam cell formation [45, 46].

In summary, HDL-associated PON1 prevents MPO-mediated HDL modification and apoAI crosslinking, which protects HDL atheroprotective functions. The increased MDA-HDL adducts in FH patients likely contribute to their decreased PON1 activity and HDL dysfunction. Inhibition of MPO generated reactive dicarbonyls with scavengers reduces HDL apoAI crosslinking and dysfunction. Finally, in vivo scavenging of reactive dicarbonyls is a novel therapeutic strategy in addition to cholesterol lowering for preserving PON1 activity, maintaining HDL function, and preventing atherosclerosis.

Abbreviations:

FH, Familial hypercholesterolemia; MPO, myeloperoxidase; PON1, paraoxonase 1; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; MDA, Malondialdehyde; HDL, high-density lipoprotein; ApoAI, ApolipoproteinA-I; ABCA1, the ATP-binding cassette transporter A1; ROS, reactive oxygen species; SAA, serum amyloid A;

Acknoledgements:

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FIGURE LEGENDS

Figure 1. Mechanism of MDA formation from peroxidation of HDL, its subsequent crosslinking of proteins and effects of reactive dicarbonyl scavenging on HDL cholesterol efflux capacity and anti-inflammatory functions.

Figure 2. PON1 inhibits MPO-mediated HDL apoAI crosslinking, decreases MDA-HDL adduct levels and preserves HDL function. MPO peroxidase activity is inhibited by PON1 as determined by an L-012 biochemical luminescence assay (A) and PON1 attenuates the crosslinking of apoAI in HDL oxidized by MPO (B). PON1 decreases the levels of MDA-HDL adducts (C) and attenuates HDL cholesterol efflux in MPO-mediated HDL oxidation (D). (B-D) Reactions were HDL alone, HDL with 160 μM H_2O_2, HDL with100 mM NaCl, 160 μM H_2O_2, and MPO, and HDL with PON1, 100 mM NaCl, 160 μM H_2O_2, and MPO. Graphs represent data (mean ± SEM) of three experiments; *P <0.05 and **P<0.01, and ***P<0.001 by one way ANOVA.

Figure 3. Effects of MDA modification of apoAI or HDL on apoAI crosslinking. (A-D) ApoAI or HDL were modified with increasing concentrations of MDA. The crosslinking of apoAI was determined in MDA modified apoAI (A) and HDL (B) by western blotting with anti-ApoAI antibody. Formation of MDA-apoAI adducts was measured in both MDA-modified apoAI (C) and MDA-modified HDL (D) by ELISA. Graphs represent data (mean ± SEM) of the experiments; *P <0.05, **P<0.01, and ***P<0.001 by one way ANOVA.

Figure 4. Effects of MDA modification on cholesterol efflux capacity, apoAI lysine availability, and HDL-apoAI exchangeability. ApoAI (A) or HDL (B) were modified with increasing concentrations of MDA and the effects on cholesterol efflux capacity were determined. The availability free lysine after MDA modification of HDL was measured using the OPA assay (C). The effects of MDA modification of HDL on apoAI

exchangeability was measured by EPR (D). Graphs represent data (mean ± SEM) of the experiments; *P <0.05, **P<0.01, and ***P<0.001 by one way ANOVA.

Figure 5. Effects of reactive dicarbonyl scavenging on HDL-apoAI crosslinking and cholesterol efflux capacity during MDA modification and MPO-mediated oxidation. ApoAI crosslinking (A) and cholesterol efflux capacity (B) were determined in HDL treated with or without 5 mM taurine before addition of MDA (0.25 mM MDA). The ability of reactive dicarbonyl scavenging with 5 mM taurine (C), 0.5 mM 2-HOBA (D), or 0.5 mM PPM (E) to prevent MPO-HDL-apoAI crosslinking was determined. The cholesterol efflux capacity of HDL modified with MPO in the absence and presence of 2-HOBA was measured (F). Graphs represent data (mean ± SEM) of the experiments; *P <0.05 by one way ANOVA.

Figure 6. Effects of HDL MDA modification and reactive dicarbonyl scavenging on PON1 activity. The effect of MDA modification of HDL on PON1 activity was measured (A). The PON1 activity was measured in plasma of *Ldlr*$^{-/-}$ mice (n=15 in control, n=13 in treatment group) consuming a western diet and treated with water alone or with the reactive scavenger, 2-HOBA (B). The cholesterol efflux capacity of HDL from *Ldlr*$^{-/-}$ mice treated with water alone or with the reactive dicarbonyl scavenger, PPM (C). MDA-HDL (D) and MDA-LDL (E) levels were measured in *Ldlr*$^{-/-}$ mice treated with the reactive dicarbonyl scavenger PPM using ELISA. Graphs represent data (mean ± SEM) of the experiments; *P <0.05 by T-test or one way ANOVA.

Figure 7. FH patients have decreased PON1 activity, increased MDA-apoAI adducts, defective HDL cholesterol efflux capacity, and impaired HDL anti-inflammatory function. The PON1 levels were determined in the ApoB-depleted serum fraction (A) from

control (n=9) and FH (n=6) subjects pre and post LDL apheresis. The PON1 activity was determined in ApoB-depleted serum fraction(B) and the specific activity of PON1 in control and FH subjects is shown (C). HDL-MDA content (D) and cholesterol efflux capacity (E) were determined in control versus FH subjects. Graphs represent data (mean ± SEM) of the experiments; *P <0.05 by one way ANOVA.

Figure 8. MDA modified HDL increases expression of pro-inflammatory genes and FH-HDL exerts remarkable pro-inflammatory properties on *Apoe*$^{-/-}$ macrophages. HDL was modified with increasing doses of MDA and the effects on macrophage expression of IL-1β (A) and IL-6 (B) in response to LPS was measured. Graphs represent data (mean ± SEM) of the experiments; *P <0.05 by one way ANOVA. IL-1β (C) and TNF-α (D) mRNA levels were measured in *Apoe*$^{-/-}$ macrophages treated with LPS and HDL from control (10) or FH subjects pre and post LDL apheresis (n=7).

Fig 1

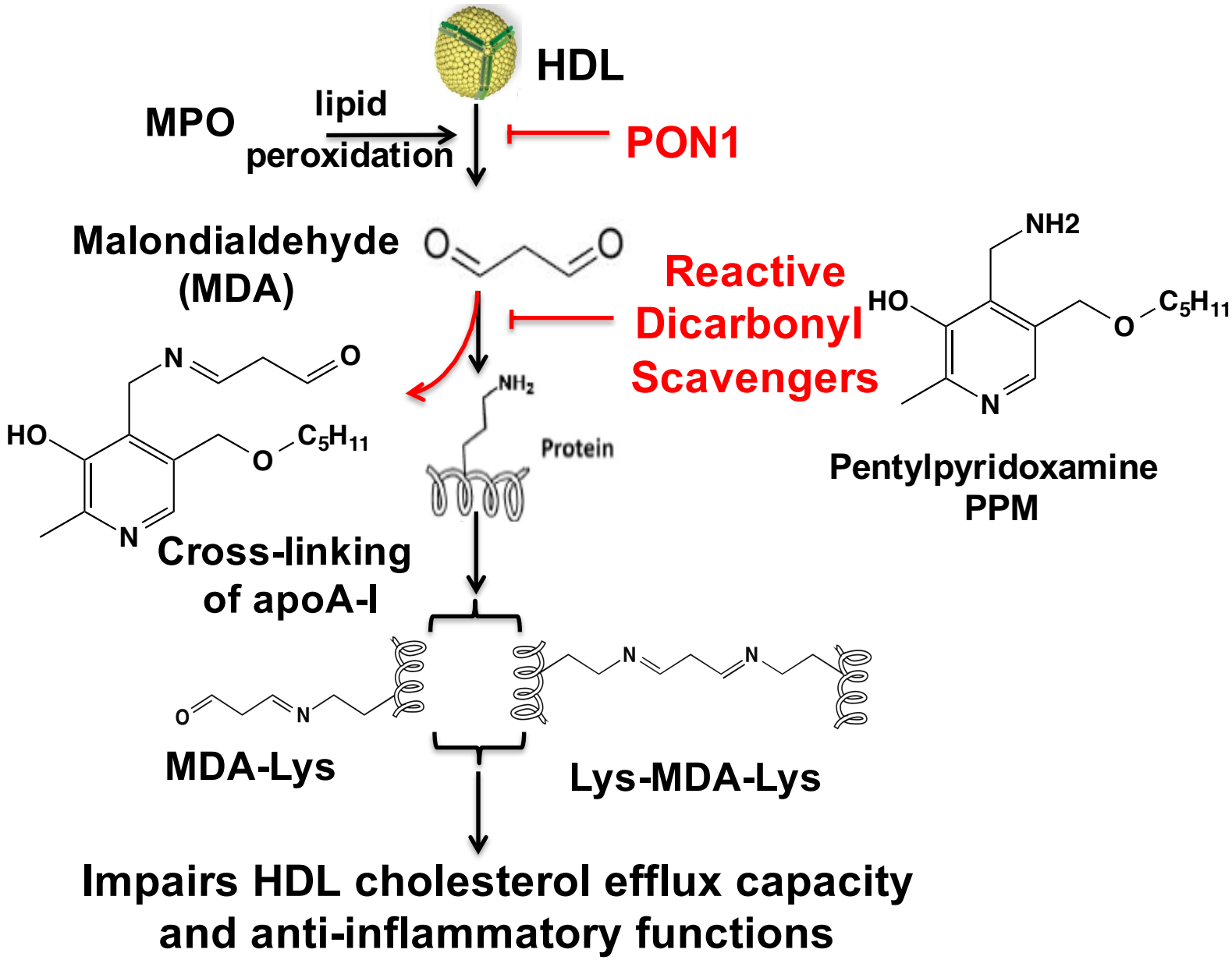

HDL
MPO
lipid peroxidation
PON1
Malondialdehyde (MDA)
Reactive Dicarbonyl Scavengers
NH2
HO
O
C5H11
N
Pentylpyridoxamine PPM
NH2
Protein
Cross-linking of apoA-I
MDA-Lys
Lys-MDA-Lys
Impairs HDL cholesterol efflux capacity and anti-inflammatory functions

Fig 2

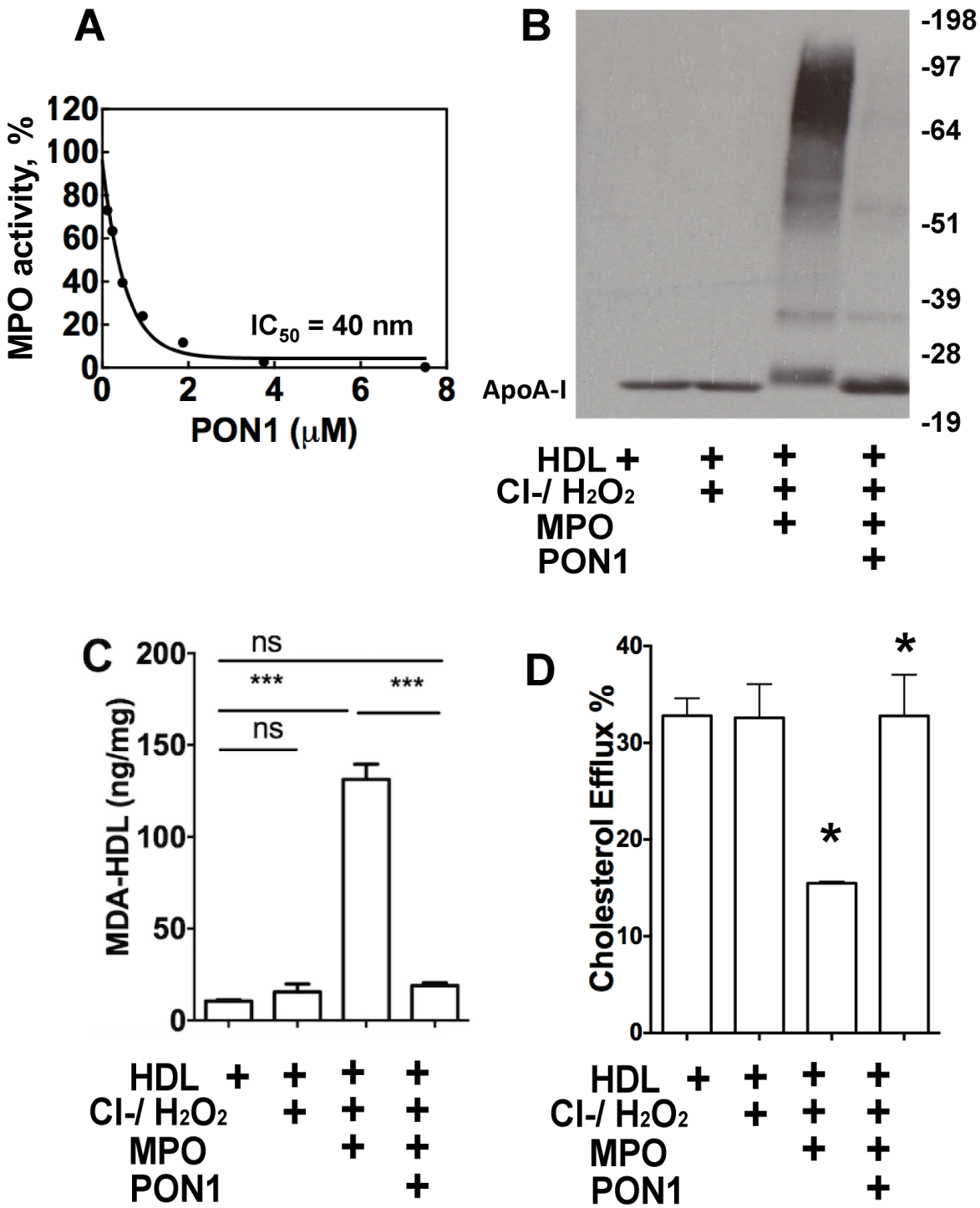

A
MPO activity, %
120
100
80
60
40
20
0
0
2
4
6
8
PON1 (μM)
IC50 = 40 nm
B
-198
-97
-64
-51
-39
-28
-19
ApoA-I
HDL + + + +
Cl-/ H2O2 + + +
MPO + +
PON1 +
C
MDA-HDL (ng/mg)
200
150
100
50
0
ns
***
***
ns
HDL + + + +
Cl-/ H2O2 + + +
MPO + +
PON1 +
D
Cholesterol Efflux %
40
30
20
10
0
*
*
HDL + + + +
Cl-/ H2O2 + + +
MPO + +
PON1 +

Fig 3

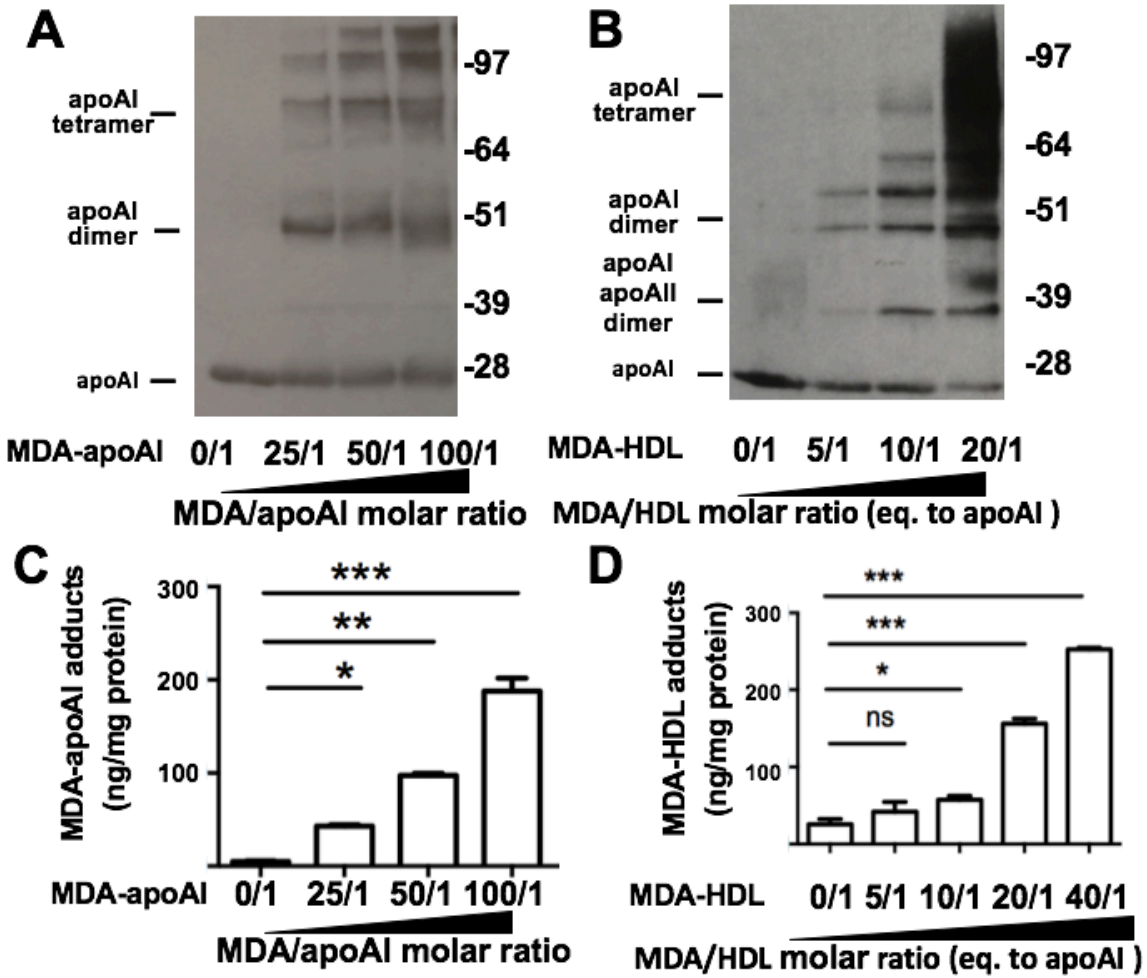

A
apoAI tetramer
apoAI dimer
apoAI
-97
-64
-51
-39
-28
MDA-apoAI 0/1 25/1 50/1 100/1
MDA/apoAI molar ratio
B
apoAI tetramer
apoAI dimer
apoAI
apoAII
dimer
apoAI
-97
-64
-51
-39
-28
MDA-HDL 0/1 5/1 10/1 20/1
MDA/HDL molar ratio (eq. to apoAI)
C
MDA-apoAI adducts (ng/mg protein)
300
200
100
***
**
*
MDA-apoAI 0/1 25/1 50/1 100/1
MDA/apoAI molar ratio
D
MDA-HDL adducts (ng/mg protein)
300
200
100
***
***
*
ns
MDA-HDL 0/1 5/1 10/1 20/1 40/1
MDA/HDL molar ratio (eq. to apoAI)

Fig 4

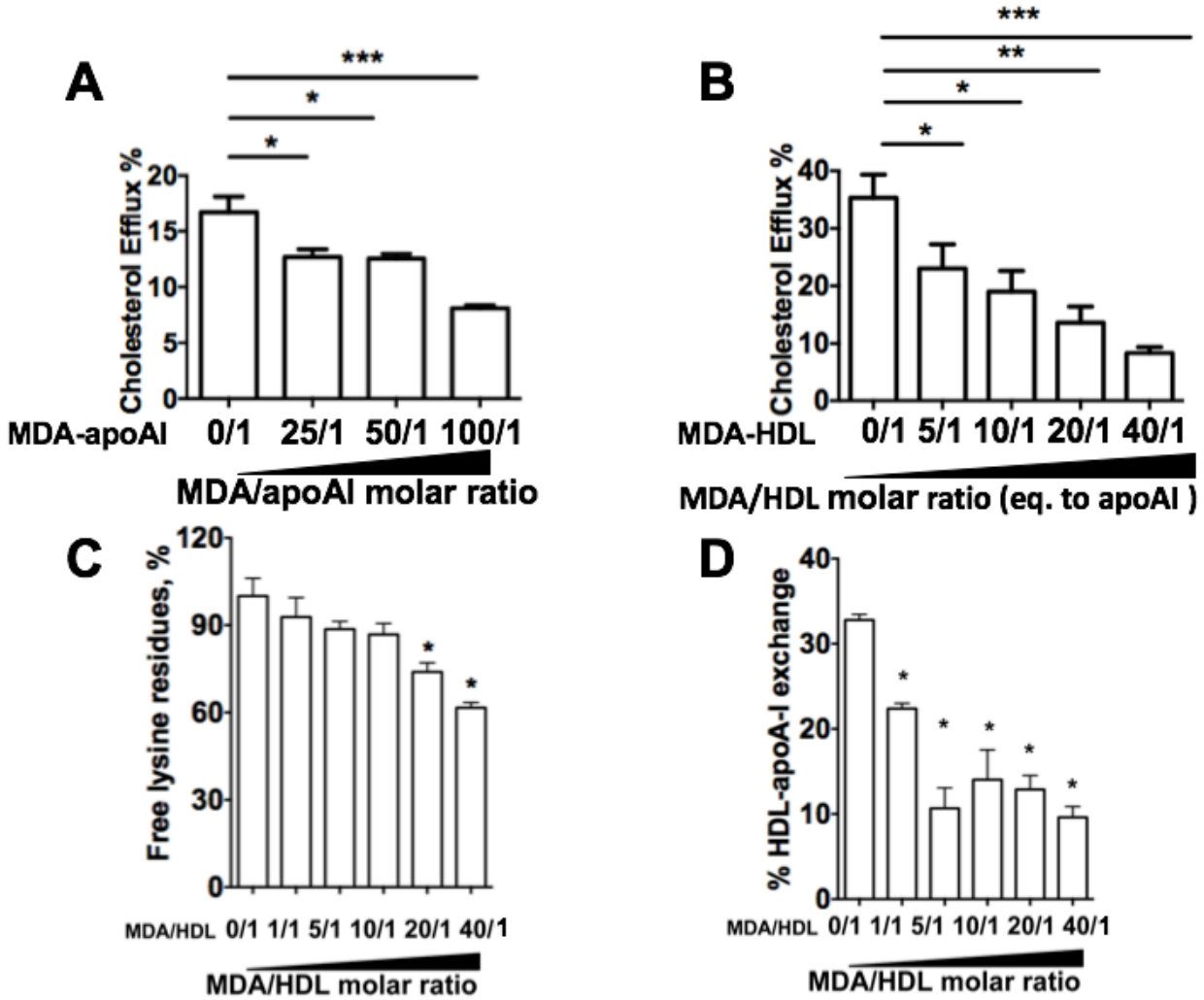

A
***
*
*
Cholesterol Efflux %
20
15
10
5
0
MDA-apoAI
0/1
25/1
50/1
100/1
MDA/apoAI molar ratio
B
***
**
*
*
Cholesterol Efflux %
40
30
20
10
0
MDA-HDL
0/1
5/1
10/1
20/1
40/1
MDA/HDL molar ratio (eq. to apoAI)
C
Free lysine residues, %
120
90
60
30
0
*
*
MDA/HDL
0/1
1/1
5/1
10/1
20/1
40/1
MDA/HDL molar ratio
D
% HDL-apoA-I exchange
40
30
20
10
0
*
*
*
*
*
MDA/HDL
0/1
1/1
5/1
10/1
20/1
40/1
MDA/HDL molar ratio

Fig 5

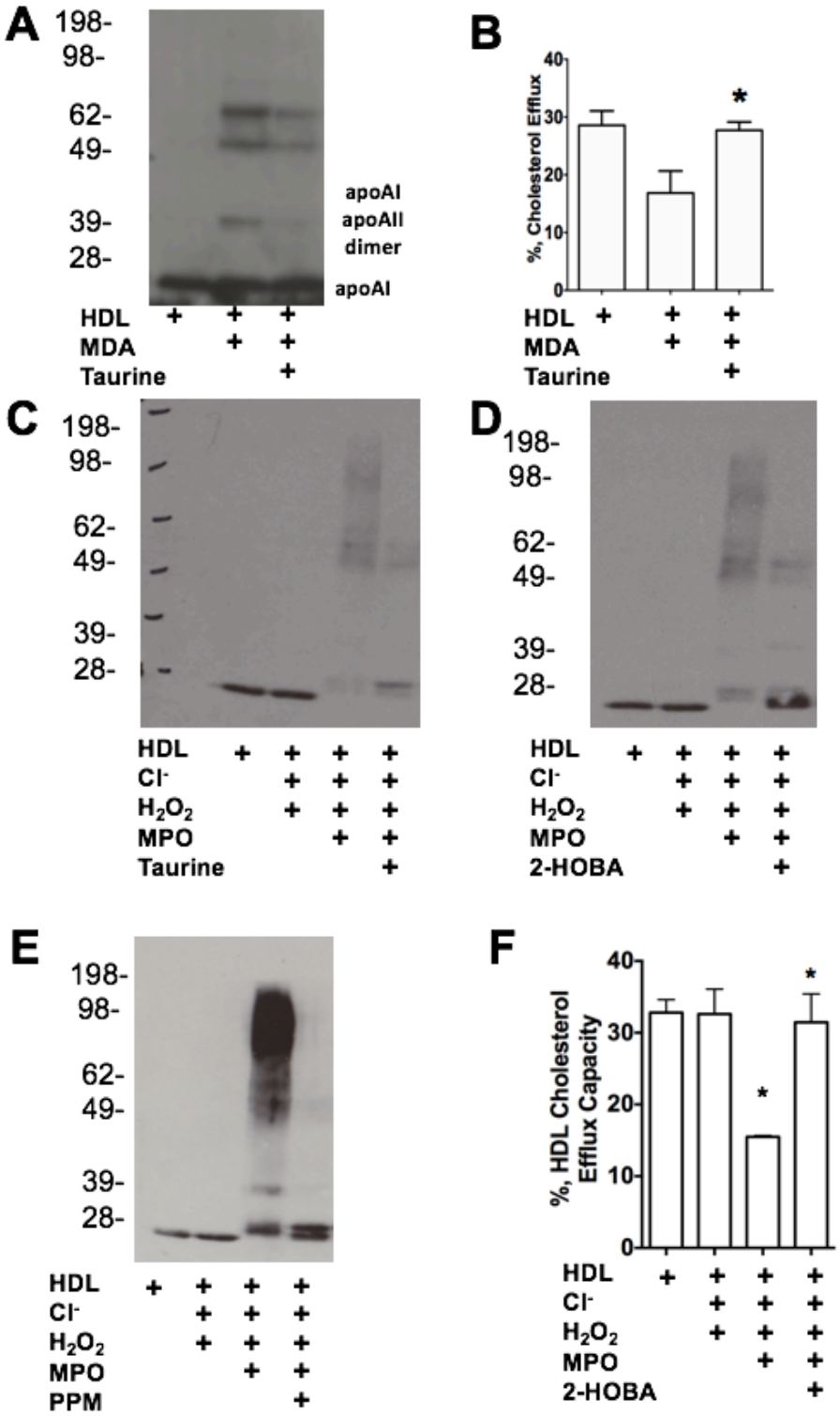

A
198-
98-
62-
49-
39-
28-
apoAI
apoAII
dimer
apoAI
HDL + + +
MDA + +
Taurine +
B
%, Cholesterol Efflux
40
30
20
10
0
*
HDL + + +
MDA + +
Taurine +
C
198-
98-
62-
49-
39-
28-
HDL + + + +
Cl- + + +
H2O2 + + +
MPO + +
Taurine +
D
198-
98-
62-
49-
39-
28-
HDL + + + +
Cl- + + +
H2O2 + + +
MPO + +
2-HOBA +
E
198-
98-
62-
49-
39-
28-
HDL + + + +
Cl- + + +
H2O2 + + +
MPO + +
PPM +
F
%, HDL Cholesterol Efflux Capacity
40
30
20
10
0
*
*
HDL + + + +
Cl- + + +
H2O2 + + +
MPO + +
2-HOBA +

Fig 6

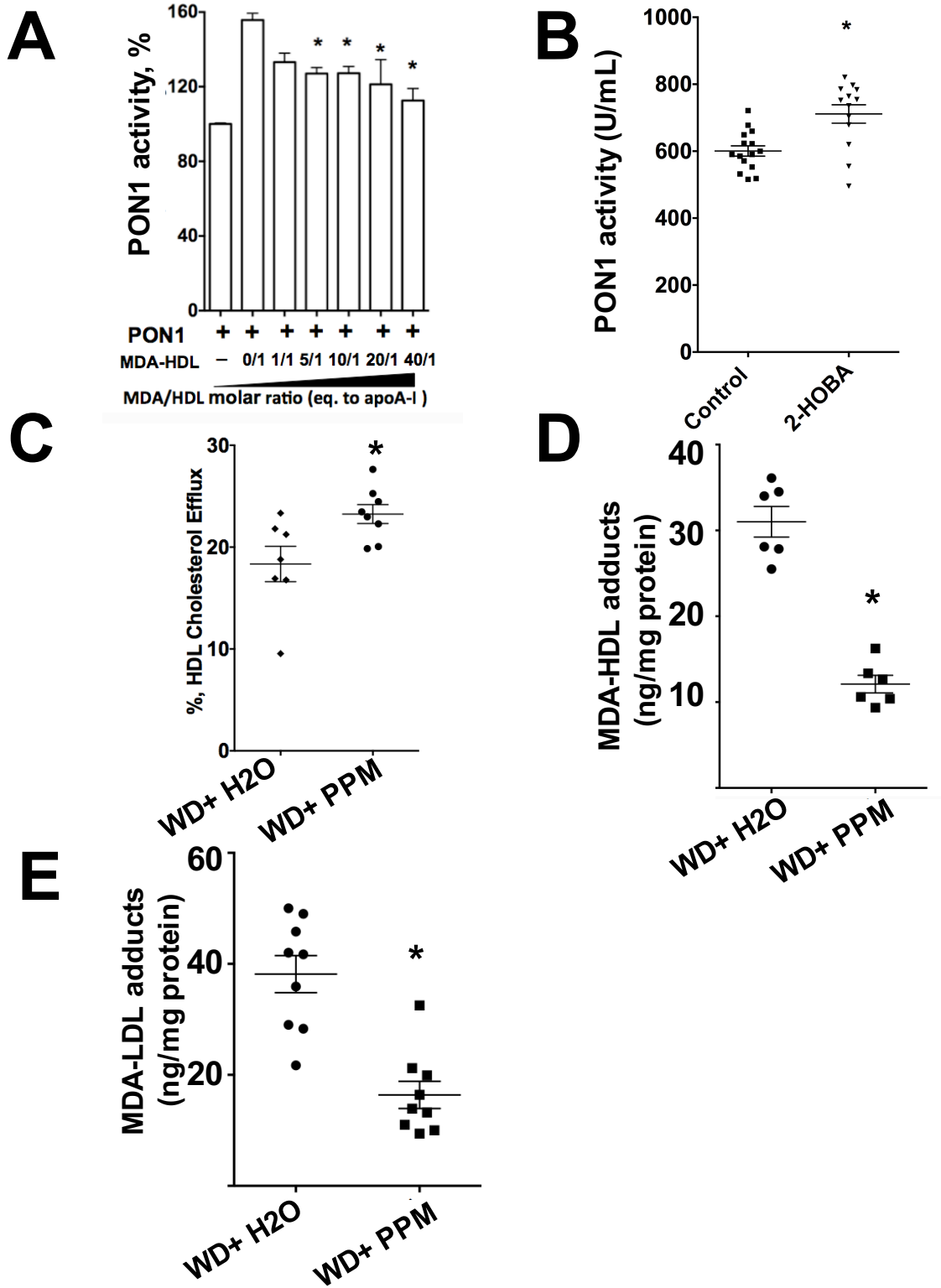

A
PON1 activity, %
160
120
80
40
0
PON1 + + + + + + +
MDA-HDL – 0/1 1/1 5/1 10/1 20/1 40/1
MDA/HDL molar ratio (eq. to apoA-I)
B
PON1 activity (U/mL)
1000
800
600
400
200
0
Control
2-HOBA
C
%, HDL Cholesterol Efflux
30
20
10
0
WD+ H2O
WD+ PPM
D
MDA-HDL adducts (ng/mg protein)
40
30
20
10
WD+ H2O
WD+ PPM
E
MDA-LDL adducts (ng/mg protein)
60
40
20
WD+ H2O
WD+ PPM

Fig 7

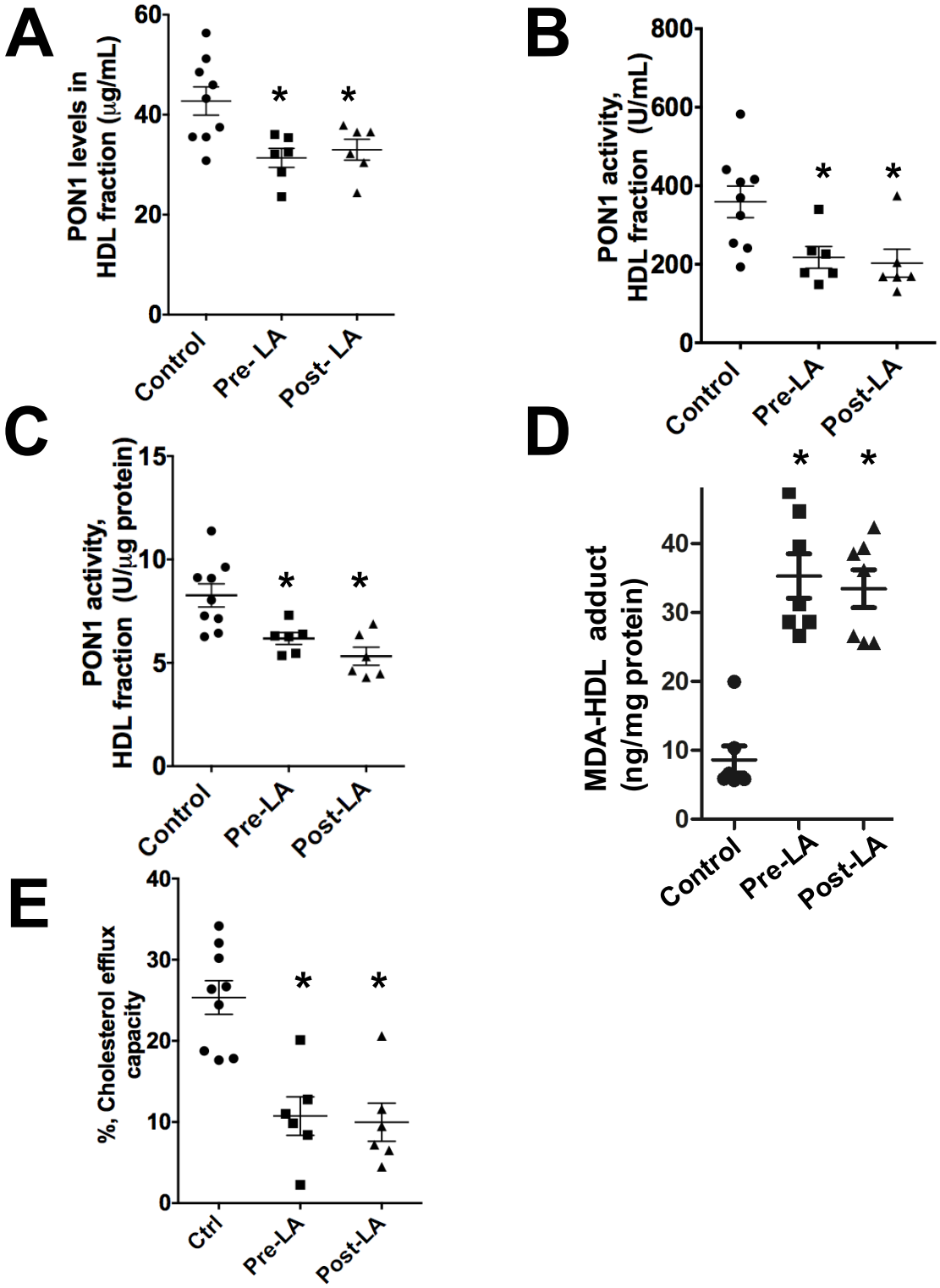
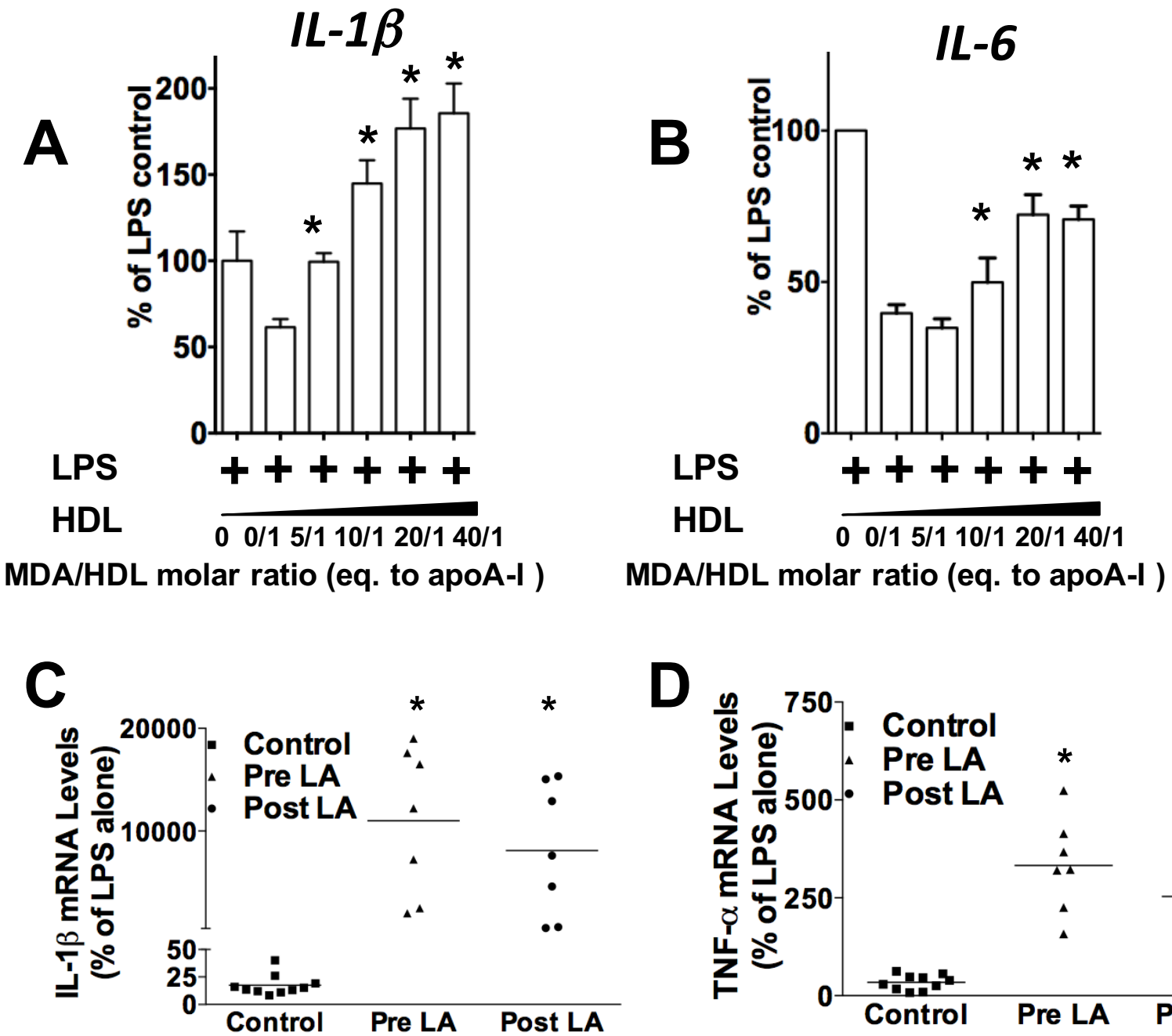

A
PON1 levels in HDL fraction (μg/mL)
60
40
20
0
Control
Pre- LA
Post- LA
B
PON1 activity, HDL fraction (U/mL)
800
600
400
200
0
Control
Pre-LA
Post-LA
C
PON1 activity, HDL fraction (U/μg protein)
15
10
5
0
Control
Pre-LA
Post-LA
D
MDA-HDL adduct (ng/mg protein)
40
30
20
10
0
Control
Pre-LA
Post-LA
E
%, Cholesterol efflux capacity
40
30
20
10
0
Ctrl
Pre-LA
Post-LA

Fig 8


IL-1β
A
% of LPS control
200
150
100
50
0
LPS
HDL
0 0/1 5/1 10/1 20/1 40/1
MDA/HDL molar ratio (eq. to apoA-I)
IL-6
B
% of LPS control
100
50
0
LPS
HDL
0 0/1 5/1 10/1 20/1 40/1
MDA/HDL molar ratio (eq. to apoA-I)
C
IL-1β mRNA Levels
(% of LPS alone)
20000
10000
50
25
0
Control
Pre LA
Post LA
Control
Pre LA
Post LA
D
TNF-α mRNA Levels
(% of LPS alone)
750
500
250
0
Control
Pre LA
Post LA
Control
Pre LA
Post LA