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Feasibility of a Plasma Bioassay to Assess Oxidative Protection of Low Density Lipoproteins by High Density Lipoproteins

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Abstract

Background: Traditionally, the impact of lipoproteins on vascular disease has been evaluated in light of their quantity, i.e. cholesterol content, in plasma. However, recent studies of high-density lipoproteins (HDL) have focused on functionality with regard to atheroprotection. For example, bioassays have emerged to assess the ability of HDL, in its near native plasma environment, to promote cholesterol removal (efflux) from cells. As a result, attention has focused on developing plasma-based assays for other putative HDL protective functions including protecting low-density lipoproteins (LDL) from oxidative damage.

Objective: To determine the feasibility of such an assay in a complex sample such as plasma, we evaluated the contribution of HDL vs. other plasma factors in preventing LDL oxidation.

Methods: We separated normolipidemic human plasma by gel filtration chromatography and assessed each fraction for its ability to prevent LDL modification by water soluble radical and copper initiated oxidation mechanisms.

Results: Using proteomics and selective precipitation methods, we identified major antioxidative contributions for fibrinogen, immunoglobulin G, albumin and small soluble molecules like uric acid and ascorbate, with albumin being especially dominant in copper initiated mechanisms. HDL particles were minor contributors (~1–2%) to the antioxidant capacity of plasma, irrespective of oxidation mechanism.

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Conclusions: Given the overwhelming background of antioxidant capacity inherent to highly abundant plasma proteins, specific bioassays of HDL antioxidative function will likely require its complete separation from plasma.

Keywords

High density lipoprotein (HDL); antioxidant; low density lipoprotein (LDL); lipid peroxidation; albumin; fibrinogen; immunoglobulin G; mass spectrometry

Introduction

Atherosclerosis is the major underlying cause of coronary artery disease (CAD) and is characterized by the accumulation of inflammatory plaques in the vessel wall. These lesions contain “foamy” macrophages that are lipid-laden via uptake of lipoproteins, primarily low-density lipoproteins (LDL). According to the oxidative modification hypothesis (1, 2), oxidized LDL is one factor thought to initiate and propagate plaque development. Indeed, macrophage scavenger receptors can take up oxidized LDL 20 times faster than native LDL uptake via the LDL receptor (3). Although the exact contribution of this pathway has been questioned by recent clinical trials showing a lack of benefit for lipid soluble antioxidants (4), oxidized LDL exhibit a host of pro-atherogenic features (5) and oxidized lipids are clearly present in the atherosclerotic lesion (6). Thus, there is interest in plasma factors that limit LDL oxidation.

Plasma levels of high-density lipoprotein cholesterol (HDL-C) are inversely correlated to CAD. Because of recent discordances between pharmacologic/genetically driven changes in HDL-C and disease outcomes (7), there has been increased emphasis on the functional evaluation of HDL particles, particularly in their plasma environment. For example, HDL can promote cholesterol efflux and subsequent transport of peripheral cell cholesterol to the liver for catabolism (8, 9). Rader et al. (10) and others (reviewed in (11)) have demonstrated that an *in vitro* derived cholesterol efflux index of apolipoprotein B-depleted serum is a more rigorous cardiovascular disease marker than HDL-C, leading to an explosion of plasma/serum based cholesterol efflux studies. Another of HDL's most established roles is its ability to protect LDL from oxidation (reviewed in (12)). HDL may also clear lipid peroxides in LDL and deliver them to the liver and/or metabolize them with resident lipolytic enzymes like paraoxonase 1 (PON1), lecithin:cholesterol acyl transferase (LCAT) or other lipases. Indeed, specific HDL subfractions have been implicated in LDL protection, suggesting that certain particles may be tailored for this function (13). Thus, it may be beneficial to evaluate HDL's oxidative properties in a plasma setting as a complement to cholesterol efflux measurements. However, the antioxidative properties of plasma are complicated. While isolated HDL particles can clearly inhibit LDL oxidation (14), potent antioxidative properties have also been described for non-lipoprotein plasma components (15) and soluble metabolites (16).

To assess the possibility of developing a laboratory assessment of HDL antioxidative function analogous to the cholesterol efflux bioassay, we set out to first understand the magnitude of HDL's effects vs. other antioxidative factors in plasma when compared at their

physiologically relevant ratios. We separated the plasma of normolipidemic human subjects by gel filtration chromatography and assessed each fraction for its ability to prevent LDL oxidation by the water soluble radical initiator, 2,2,- Azobis (2-methylpropionamidine) dihydrochloride (AAPH) (17) and by copper mediated oxidation. We found that HDL particles were relatively minor contributors to the antioxidant capacity of plasma in both oxidation models with the major players being abundant soluble proteins and small metabolites.

Materials and Methods

Human Subjects and Plasma Collection –

Fasting plasma was obtained using sodium citrate (BD Vacutainer tubes) as an anticoagulant from healthy, normolipidemic males whose BMI was less than 24.9. Plasma was subjected to gel filtration chromatography, within 2 h of collection. Human subjects provided informed consent according to an approved protocol overseen by the institutional review board at Cincinnati Children's Hospital Medical Center. Plasma deficient in fibrinogen, apoH, plasminogen or factor X were purchased from Affinity Biologicals (Ontario, Canada). Human fibrinogen, human IgG and human serum albumin were purchased from Sigma-Aldrich (St. Louis, MO).

Plasma Fractionation –

Plasma (354 µl) was fractionated on three Superdex 200 columns (10/300 GL; GE Healthcare Lifesciences, Pittsburgh, PA) as previously described (18, 19). Plasma fractions (1.5 ml) were stored at 4 °C and used within a week of fractionation. Some plasma samples were passed over a G-25 spin column prior to fractionation, depending on the experimental goals. Briefly, 500 µl of plasma was passed through an equilibrated G-25 column. An extra 250 µl STB- (10 mM Tris, 0.15 M NaCl, pH 8.2) was applied for complete elution of all plasma proteins and phospholipids. For direct comparison with G-25 depleted plasma, control plasma was equivalently diluted by 33%. In other cases, as noted, plasma was treated with Lipid Removal Agent (LRA, Supelco, Bellefonte, Pennsylvania) or CleanAscites™ (CA, Biotech Support Group, Monmouth Junction, NJ) prior to fractionation to remove lipid bound proteins, or heparin/manganese (Mn) to remove apoB containing particles prior to fractionation. For LRA or CA treatment, 250 µl plasma was diluted 1:1 and then incubated for 30 min with 200 µl of an LRA suspension (100 mg/ml) or 500 µl of CA suspension. LRA or CA were pelleted by centrifugation and the plasma was recovered and filtered prior to injection on the gel filtration columns. For apoB depletion, plasma (330 µl) was incubated with heparin (13.2 µl of 35.4 mg/ml heparin) and 16.5 µl of 1M MnCl₂ for 30 min. The apoB containing particles were pelleted by centrifugation for complete removal (20, 21). The resulting plasma was diluted up to 660 µl resulting in a 1:1 diluted apoB depleted plasma sample. The LRA, CA or heparin depleted, or control plasma (1:1 diluted) samples were then subjected to fractionation over the three Superdex columns, as described above. Albumin was depleted from plasma using a Cibacron blue column (CaptoBlue, GE Healthcare Lifesciences). Cholesterol and phosphatidylcholine containing phospholipid (PL) content of the fractions were analyzed using enzymatic kits (Pointe Scientific, Canton, MI and Wako, Richmond, VA). Protein content was measured using a modified Lowry assay

(22). Purified human serum albumin (60 mg/ml), or human immunoglobulin (10 mg/ml) samples were fractionated similarly to plasma samples. The resulting fractions were compared with plasma fractions within two days of each other.

LDL Oxidation Assay –

LDL oxidation was analyzed by measuring the production of conjugated dienes as previously described (19). Briefly, LDL isolated from a single donor by ultracentrifugation (20 µg of LDL-C) was pre-incubated with 50 µl of each fraction at 37 °C for 10 min. prior to initiation of oxidation by the addition of 5 mM (final concentration) 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH, Acros Organics, Geel, Belgium) or 2.5 µM copper sulfate (final concentration). Conjugated diene production was measured at A₂₃₄ for 8 h at 37 °C. LDL oxidation was compared using the propagation rate (PR) of the reaction. Propagation rate represents the maximal slope (V_{max}) of oxidation. Percent inhibition is calculated by the decrease in the PR as compared to LDL only. Component peaks of the resulting antioxidation curve across fractions were deconvoluted using Peakfit software (Systat software, v. 4.12) using the residual best fit method. Although our focus was on PR, we also analyzed lag time and obtained results similar to PR.

Mass Spectrometry Sample Preparation and Analysis –

Plasma fractions were prepared for MS, as previously reported (19, 23). Mass spectrometry (MS) was performed on trypsinized proteins using a nanoLC-MS/MS (AB Sciex5600 +TripleTOF) mass spectrometer as previously described (19, 23). Mascot (version 2.2.2) and X! Tandem (version 2001.01.01.1) search engines were used to identify proteins, and were searched against the UniProtKB/Swiss-Prot Protein Knowledgebase (release 57.0, 03/2009) using human taxonomy. Carbamidomethylation was a variable modification; peptide tolerance was set at ±20 ppm, MS/MS tolerance set to ± 0.6 Da, and up to 3 missed trypsin cleavages were allowed. Scaffold software (version 3.3.1) was used for validation of peptide and protein identifications from the MS/MS and only peptides and proteins with >99% identification probability were included in the analysis. For each protein to be considered in the analysis, the identification of at least 2 peptides were required. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (24) partner repository with the dataset identifier PXD006083 and 10.6019/PXD006083.

Statistical Analysis.

All data are expressed as mean ± standard deviation unless otherwise specified. Oxidation experiments were performed using technical duplicates or triplicates, depending on the experiment. In all cases, experiments were repeated at least twice.

Results

Plasma Profile of LDL Oxidative Protection.

To understand the plasma components that contribute to its antioxidative capacity, we took an 'old-school' biochemical approach. Plasma samples obtained from 10 normolipidemic humans were analyzed by gel filtration. Each resulting fraction was assessed (at equal volume) for the ability to inhibit AAPH or copper mediated oxidation of exogenously added

human LDL. In characterizing this separation system, we have established that all of the protein, phospholipid (PL) and cholesterol (CH) elute between fractions 13–31 (18, 19, 25), though small metabolites and salts elute as late as fraction 48. In initial experiments, we ran the separation past fraction 31 to get an idea of the contribution of small soluble metabolites. For AAPH oxidation, an anti-oxidation activity peak was observed at fraction 46, accounting for about 6% of the total anti-oxidant activity (Supplement Fig. 1a). This was likely due to small molecule metabolites, probably uric acid and/or ascorbate, because the peak disappeared when plasma was passed through a G25 desalting column prior to the separation (Supplement Fig. 1b). Because the antioxidant activities of small molecules have been extensively studied (26), we focused on the protein and lipid containing species eluting between fractions 13–31 for this work.

The antioxidant activity profile across the protein/lipid containing fractions using AAPH (Fig. 1a) or copper (Fig. 1b) are shown. The dashed or dotted traces show the peak deconvolution analysis (see *Methods*) revealing fits to each activity in isolation. For AAPH catalyzed oxidation, we identified three distinct activity peaks. For copper, we observed two clear peaks that corresponded with peaks 1 and 3 in the AAPH experiment. In the case of copper, the first peak was relatively minor compared to the second. Fig. 1c shows examples of oxidation kinetics obtained in fractions from each major peak (from the AAPH experiments). The maximum slope of each curve was defined as the propagation rate (PR) which we used as the activity measure. In buffer only, LDL was rapidly oxidized by AAPH. The addition of the peak fractions 18, 25, and 29 slowed the PR to various extents.

The position of each antioxidation peak (again using the AAPH data as an example) with respect to total cholesterol, phospholipid or protein is shown in Fig. 2. Antioxidation peaks 1 and 2 roughly corresponded to CH and PL peaks whereas peak 3 did not overlap significantly with the lipids. The elution ranges of apoB (VLDL and LDL), apoA-I (HDL) and human albumin are shown for reference (19, 25). All three activity peaks corresponded with peaks in total protein (Fig. 2c). We set out to identify the plasma components responsible for each peak for both AAPH and copper mediated oxidation.

Identity of AAPH Peak 1.

Fig. 2 shows that the activity in peak 1 contains PL, CH and protein. It also roughly corresponded with the elution of apoB containing particles, LDL and VLDL (18,19). Thus, we started with the simplest hypothesis that the antioxidant activity in peak 1 was due to endogenous VLDL/LDL. To test this, we depleted plasma of phospholipid containing particles with two phospholipid affinity resins: lipid removal agent (LRA) (18) and Cleanascite™ (CA) (27). These bind phospholipids tightly via distinct non-covalent mechanisms. We also treated plasma with heparin/manganese (Mn) under conditions that quantitatively precipitate VLDL and LDL, but not HDL (20). After depletion, the plasma samples were size separated and each fraction assessed for activity against LDL oxidation by AAPH. Fig. 3 shows that plasma pretreatment with both resins largely ablated the antioxidant activity in fraction 18 (peak 1). This suggests that the putative antioxidants either *i)* contain phospholipid, or *ii)* non-specifically bind to both resins. However, heparin/Mn treatment failed to decrease the antioxidant activity in peak 1, despite the quantitative

depletion of apoB (not shown). This argued against the idea that endogenous plasma LDL or VLDL was responsible for the peak 1 activity. To confirm this, we size fractionated ultracentrifugally isolated human LDL starting with similar levels of total PL as found in plasma. Despite eluting at a similar time and in similar amounts to plasma (Fig. 4a), isolated LDL did not recapitulate the antioxidant activity observed in peak 1 of human plasma (Fig. 4b). Since these results argued against a major role for VLDL or LDL in peak 1, we searched for other proteins that correlated strongly with the antioxidant activity. Candidates would have to bind to LRA and CA, but not be affected by heparin/Mn. In previous work (19), we performed mass spectrometry (MS) to identify proteins contained in gel filtration fractions (13–31) from 10 normolipidemic participants. Supplemental Fig. 2 shows a heat map for proteins whose separation pattern roughly corresponded to the LDL antioxidative activity in peaks 1–3. We correlated the spectral counts for each protein across these fractions with LDL antioxidation activity peaks using a Pearson correlation analysis. Table 1 shows the proteins that exhibited correlation coefficients above 0.90, ranked by known abundance in human blood. We reasoned that changes in the levels of these candidate factors should mirror the antioxidation activity when treated with the PL binding resins and heparin/Mn and, in that case, residuals would be small.

Fig. 5a shows the LDL antioxidant activity in peak 1 before (NT) and after plasma was treated with LRA, CA or heparin/Mn. The peak 1 (fraction 18) antioxidant activity was set to 100% in untreated plasma. Plasma pretreated with the PL binding resins LRA or CA exhibited little measurable activity. However, heparin/Mn treated plasma retained ~95% of antioxidant activity in peak 1. We then compared the spectral counts for each candidate protein in Table 1 after each treatment vs. antioxidant activity. Fig. 5a shows that fibrinogen protein abundance levels (measured by MS peptide counting) varied similarly to the antioxidant activity of plasma across the various treatments. α 2 macroglobulin (A2MG) also displayed a similar pattern but the correlation (as determined by the residuals) was less strong. ApoB levels did not track with the activity consistent with the results above.

The experiments in Fig. 5a pointed to fibrinogen being responsible for antioxidant activity peak 1. Follow-up work demonstrated that fibrinogen associates with LRA and CA (not shown), supporting this hypothesis. We obtained fibrinogen-depleted plasma (Affinity Biologics, defibrinated using purified human thrombin) and compared its LDL antioxidant profile with normal plasma. Fig. 6 shows that fraction 18 (peak 1) of fibrinogen-depleted plasma largely lacked the antioxidant activity of normal plasma. Furthermore, when we spiked purified fibrinogen back into fibrinogen-deficient plasma at physiological levels, we rescued a majority of the antioxidant effect. Thus, we concluded that most of the AAPH LDL antioxidant activity in peak 1 is due to fibrinogen.

Identity of AAPH Peak 2.

Like peak 1, peak 2 overlaps with PL, CH and protein (Fig. 2). Since the peak substantially overlapped with apoA-I, and given the plethora of literature demonstrating the antioxidative functionality of HDL, we hypothesized that the peak 2 activity was mostly contributed by HDL. To test this, plasma was incubated with LRA, CA or heparin/Mn, separated by gel filtration, and the fractions were analyzed for LDL antioxidant activity. Fig. 7a shows that

both the LRA and CA phospholipid binding resins depleted the antioxidant activity to a surprisingly small extent (~20% for LRA and ~25% for CA). Heparin/Mn treatment had no effect as expected because apoB containing lipoproteins do not elute in this peak. This suggests that the majority of the antioxidant activity in peak 2 was not associated with phospholipids, i.e. HDL. To confirm this, we size fractionated ultracentrifugally isolated human HDL, loading equal PL content to that found in plasma. When we analyzed the fractions that corresponded to peak 2, UC isolated HDL could only account for a small fraction (maximum of 5%) of the activity found in plasma peak 2 (Fig. 7b).

We were concerned that the antioxidant potential of HDL was artifactually minimized under the conditions of our assay. Therefore, we repeated this experiment using a range of AAPH concentrations from 0 – 5 mM (Supplementary Fig. 3). At extremely low levels of AAPH, we observed a small but statistically significant difference between unmodified plasma and that depleted with LRA. However, this was still consistent with a minor role for HDL under these conditions (note: there were no differences in the HDL contribution to copper-mediated oxidation, even at very low concentration). We worried that gel filtration separation of HDL may modify HDL or remove some key factor responsible for antioxidant activity. Therefore, we directly compared UC isolated HDL (not passed down a column) with fraction 25 at equivalent phospholipid concentrations. Again, UC-HDL exhibited only 6% of the antioxidant capacity of fraction 25 (Supplemental Fig. 4). Therefore, we set out to identify the additional factor(s) responsible.

Our proteomic analysis (19) revealed several proteins that correlated with the antioxidant activity of peak 2 (Table 1). We further narrowed this list by looking for proteins that did not significantly change in abundance when incubated with LRA, CA or heparin/Mn. Fig. 5b shows that human IgG was the most abundant plasma protein that fit these criteria. We obtained purified IgG and analyzed it on our gel filtration system. It eluted at a retention time that was identical to the peak 2 activity. Furthermore, Fig. 8 shows that physiological levels of IgG could account for most of the antioxidant activity in peak 2. Thus, we concluded that soluble IgG contributed ~80% of the antioxidant activity found in peak 2 of plasma, while approximately 5% of peak 2 antioxidant activity can be attributed to HDL.

Identity of AAPH Peak 3.

Fig. 2 shows that activity peak 3 was largely devoid of PL and CH. When plasma was pretreated with LRA, CA or heparin/Mn the antioxidation activity of peak 3 was unaltered (data not shown). Our proteomic analysis (19) identified three proteins whose size distributions were well correlated with the antioxidative activity in peak 3 (Table 1). Of these, albumin was by far the most abundant. Therefore, we depleted human plasma of about 90% of its albumin by passage down a Cibacron blue column (37). After separation by gel filtration, albumin depleted plasma lost most of its antioxidative activity in peak 3 (Fig. 9). Furthermore, when physiological concentrations of purified human albumin in buffer (60 mg/ml) was size fractionated, it eluted at a volume consistent with peak 3 and these fractions exhibited a similar magnitude of AAPH LDL antioxidant activity as that found in plasma (Fig. 9). Thus, we concluded that the majority of the antioxidant activity observed in peak 3 was due to human serum albumin.

Identity of Copper Peaks 1 and 3.

Fig. 1 shows that the antioxidation profile for copper induced LDL oxidation was simpler than that for AAPH containing two distinct activity peaks that corresponded in size to peaks 1 and 3 of the AAPH experiment. Given this close concordance in size, we started with the simplest hypothesis that copper Peak 1 was due to fibrinogen as it was for AAPH. Similarly, we hypothesized that copper Peak 3 was due to albumin. Using fibrinogen-deficient plasma, we found that the copper antioxidant capacity of fraction 18 was reduced by ~60% compared to normal plasma (Fig. 10a). Similarly, the depletion of albumin from plasma using a CaptoBlue column reduced the copper antioxidant capacity of fraction 28 by 85% (Fig. 10b). These data strongly suggest that fibrinogen and albumin are responsible for the majority of the copper antioxidant activity in peaks 1 and 2, respectively.

Discussion

Wayner et al first used the AAPH oxidation system to quantitatively evaluate the antioxidant potential of human plasma (17). They identified uric acid and vitamin E as important players, but concluded that up to 73% of plasma antioxidant activity was associated with 'protein and lipoprotein material' as separated on a Sephadex G-200 sizing column. In this work, we identified 5 major antioxidative activities in human plasma (Supplement Fig. 1a with the identification of at least two activities in peak 2), four of which elute in the protein and lipoprotein ranges. A peak integration analysis indicated that ~15% of activity is contributed by fibrinogen, ~20% by IgG, ~1–2% by HDL, ~40% by albumin and ~6% by small molecules (Table 2).

In the case of copper mediated LDL oxidation, fibrinogen and albumin were the dominant activities. Whether these highly abundant proteins are in fact true antioxidants or simple competitors for LDL as a substrate awaits further mechanistic work.

Many reports show HDL is an effective inhibitor of LDL oxidation *in vitro*. In separate studies, we have clearly demonstrated that ultracentrifugally isolated HDL, at concentrations much higher than found in our gel filtration fractions, can prevent LDL oxidation in line with numerous previous studies (13, 38) (Swertfeger and Davidson, unpublished). However, by using equal volumes of gel filtration fractions, we were able to compare the relative magnitudes of various plasma antioxidation activities at the same ratios that they exist in native plasma. By combining this with the proteomic abundance analysis and protein precipitation techniques, we identified each factor and ranked the magnitude of each activity. We were surprised that HDL plays such a minor role with respect to other plasma components in the AAPH and copper oxidation systems. However, there are several important caveats to our experimental design. First, we examined only two of the widest recognized LDL oxidation mechanisms. Of course, there are other relevant modes of LDL oxidation including cell based oxidation (39), myeloperoxidase (40), and other mechanisms (41). We cannot rule out a larger role for HDL in these situations. For example, oxidative processes that lead to lipid hydroperoxides may be particularly susceptible to reduction by HDL. Mashima et al (42) showed that apoA-I and apoB have the ability to reduce lipid hydroperoxides whereas other plasma components were not major contributors. Second, our experiments represent a snapshot of antioxidant activity over a limited time. Our

experiments cannot assess the potential ability of HDL to act as a regenerating antioxidant, perhaps in a sequestered cellular environment for example. Lipid hydrolytic enzymatic activities such as paraoxonase 1 (43), glutathione peroxidase (44), LCAT (45) or platelet-activating factor acetylhydrolase (46) in HDL may actively metabolize lipid hydroperoxides. Thus, HDL may be a more durable antioxidant that can function long after other antioxidants have been depleted. Interestingly, we did not find a strong correlation with antioxidant activity and PON1, LCAT or glutathione peroxidase (GPX3) abundance in our study (Supplemental Fig. 5). Finally, our experiments did not account for differential rates of diffusion/access to limiting biological compartments. Large molecules like fibrinogen, likely cannot enter or exit sequestered environments like the thickened intima of major arteries as rapidly as smaller species like HDL or its apolipoproteins (47).

Nevertheless, our results highlight the challenges of developing an *in situ* assay for HDL antioxidant functionality analogous to the plasma cholesterol efflux assays that are currently popular. In most iterations of this assay, interfering LDL-mediated cholesterol efflux is removed by a precipitation step prior to the efflux assay (10). Given that HDL represents a small minority of the overall antioxidant activity in plasma, it is difficult to imagine a practical way to deplete the other significant activities in order to assess HDL antioxidant function, even with some of the other mechanisms discussed above where HDL may play larger roles. Thus, it seems the best way to assess HDL antioxidant activity is by studying it in isolation, provided one is cognizant of potential artifacts resulting from the separation procedure.

Conclusion

Our work has identified 4 high abundance plasma proteins that play significant roles in the protection of LDL from oxidation by the free radical generators AAPH and copper. We noted that HDL, a lipoprotein widely reported to perform this function, is a relatively minor player in the context of these factors in the plasma environment. Thus, the development of plasma-based bioassays that specifically measure HDL's ability to protect against LDL oxidation faces significant challenges.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

AAPH	2,2,- Azobis (2-methylpropionamidine) dihydrochloride
CA	Clean Ascites

LRA	Lipid Removal Agent
PON1	paraoxonase 1
RCT	reverse cholesterol transport
PL	phospholipid
PR	propagation rate
CH	cholesterol
UC	ultracentrifugation
GF	gel filtration

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Figure 1. LDL oxidation inhibition profile of human plasma.

Inhibition of **a)** AAPH-induced or **b)** copper-induced LDL oxidation by equal volumes of each fraction of SEC-separated human plasma. Percent inhibition (solid black circle and lines) reflects the decrease in the oxidation propagation rate (PR) as compared to LDL in buffer only. The dotted traces and shading (**a** and **b**) shows a curve peeling analysis (see *Methods*) for each peak. The data was derived from 10 normal subjects, measured in duplicate, for AAPH-induced oxidation, and 4 normolipidemic subjects, measured in triplicate for copper-induced oxidation ± 1 sample standard deviation. **c)** Examples of LDL oxidation kinetics (by AAPH) of selected plasma fractions. LDL oxidation was measured as the accumulation of conjugated dienes, reflected by an increase in absorbance at 234 nm. Shown are LDL alone (solid line), fractions 18 (peak 1, dotted line), 25 (peak 2, short dashed line), and 28 (peak 3, dashed and dotted line).

Figure 2. Overlap of cholesterol (a), phospholipid (b) and protein (c) profiles with antioxidant activity of gel filtration plasma fractions (AAPH).

The CH, PL and protein content (open circles) of each fraction was overlaid with the antioxidant activity (filled circles) of each fraction using AAPH as the oxidation initiator. All data represent the average obtained from 10 normolipidemic subjects $\pm$ 1 sample standard deviation, with the exception of the protein measurements, which represent the average and standard deviation from 3 subjects.

Figure 3. The effect of selective precipitation methods on inhibition of LDL oxidation by AAPH in peak 1.

Plasma samples were depleted of phospholipid by LRA or CA, or of apoB containing lipoproteins by heparin/Mn. The depleted plasma was then separated by gel filtration and appropriate fractions analyzed for inhibition of LDL oxidation vs. non-treated plasma. Fraction 18 (peak 1) is shown. The data was derived from a triplicate measurement and the error bars represent 1 S.D. The experiment was repeated twice using different plasma samples. * $p < 0.01$ compared with non-treated or heparin depleted plasma.

Figure 4. Effect of size fractionated UC-LDL on LDL oxidation.

Plasma or LDL isolated by ultracentrifugation (at a PL mass matched to LDL-PL mass in normal plasma) was size fractionated and appropriate fractions were assayed for inhibition of LDL oxidation initiated by AAPH. **a)** Phospholipid trace of plasma fractions (filled circles) compared with UC-LDL (open circles). **b)** Inhibition of LDL oxidation by plasma fractions (filled circles) compared with UC-LDL (open circles). Mean and S.D. from triplicate measurements are shown. Plasma fractions were measured in duplicate, so the error bars show an average standard deviation of the UC-LDL fractions. The experiment was repeated twice.

a

	NT	LRA FT	CA FT	Hep / Mn residuals		
Activity	100.0	5.9	1.5	96.5	0.0	Antioxidant activity
FIBG	100.0	0.0	0.0	90.4	13.5	Protein abundance (peptide counts)
FIBB	100.0	0.0	0.0	81.8	22.1	
FIBA	100.0	0.0	0.0	65.8	38.1	
A2MG	100.0	0.0	40.4	99.0	47.4	
APOB	100.0	0.0	0.0	0.0	103.9	

b

	NT	LRA FT	CA FT	Hep / Mn residuals		
Activity	100.0	80.4	68.9	118.2	0.0	Antioxidant activity
IGHG2	100.0	81.4	100.0	110.0	40.4	Protein abundance (peptide counts)
IGHA1	100.0	66.7	100.0	66.7	96.4	
IGHG4	100.0	0.0	80.3	111.5	98.6	
IGHG1	100.0	39.6	50.8	69.0	108.1	
IGKC	100.0	16.7	30.4	83.3	137.1	
ITIH4	100.0	0.0	0.0	155.6	186.5	
SAMP	100.0	0.0	100.0	28.6	201.2	
APOA2	100.0	0.0	0.0	35.3	232.2	

Figure 5. Relationship of antioxidant activity with MS spectral counts after various precipitation treatments.

The residual is the sum of the absolute differences between antioxidant activity and peptide counts. **a)** Antioxidant activity and peptide counts of various candidate proteins in peak 1 (fraction 18). In each case, plasma was not treated (NT), incubated with LRA (LRA), CleanAscite (CA) or Heparin/Mn and then equal volumes were size fractionated. NT plasma was set to 100% (green) and the results were scaled with red representing zero activity and yellow representing 50%. **b)** Antioxidant activity and peptide counts of various candidate proteins in peak 2 (fraction 25).

Figure 6. Effect of manipulating plasma fibrinogen levels on LDL antioxidant activity in Peak 1. Plasma was fractionated by gel filtration. Fraction 18 was compared to identically treated fibrinogen-deficient plasma (Fb-) or Fb-plasma spiked with purified fibrinogen (2 mg/ml) prior to fractionation. Mean activities of fraction 18 (peak 1) are shown from triplicate measurements $\pm$ 1 S.D. The experiment was repeated twice. * $p < 0.0001$ Fb- compared with both normal and Fb add back.

Figure 7. The effect of various precipitation methods on inhibition of LDL oxidation activity in peak 2.

a) Plasma samples were depleted of phospholipid (by LRA or CA), or of apoB containing lipoproteins using heparin/Mn. Inhibition of LDL oxidation by fraction 25 were compared with non-treated plasma. Mean values from peak 2 fractions are shown $\pm$ 1 S.D. **b)** HDL isolated from human plasma by ultracentrifugation was size fractionated at matched PL values to that of normal plasma. Antioxidant activity was measured in the LDL oxidation assay. Mean values from peak 2 (fraction 25) are shown $\pm$ 1 S.D. The experiment was repeated twice. * $p < 0.001$ compared with plasma.

Figure 8. Inhibition of LDL oxidation by plasma or purified human IgG after size fractionation. Purified human immunoglobulin G (10 mg/ml) was size fractionated in buffer along with normal plasma. The resulting fractions were compared to plasma fractions for the antioxidation activity. Mean values from peak 2 (fraction 25) are shown $\pm$ 1 S.D. The experiment was repeated twice. * $p < 0.05$

Figure 9. Effect of manipulating plasma albumin levels on LDL antioxidant activity in Peak 3. Normal human plasma was fractionated by gel filtration and peak 3 was compared to plasma that had been passed down a CaptoBlue albumin affinity column to deplete albumin prior to size fractionation, or size fractionated purified human albumin. Mean activities of peak 3 are shown from triplicate measurements. Error bars represent 1 sample standard deviation. The experiment was repeated twice. * $p < 0.001$ compared with plasma.

Figure 10. Effect of manipulating plasma fibrinogen or albumin levels on copper-mediated LDL antioxidant activity.

Plasma was fractionated by gel filtration and equal volume of fractions were analyzed for their ability to inhibit 2.5 μM CuSO_4 mediated LDL oxidation, as measured by PR. **a)** Fraction 18 from normal plasma was compared to identically treated fibrinogen-deficient plasma (Fb -). **b)** Fraction 29 from normal plasma or plasma depleted of albumin using a CaptoBlue affinity column was analyzed for their ability to inhibit copper-mediated LDL oxidation. Mean activities of fraction 18 (peak 1, **a**) or peak 3, **b**) are shown from triplicate measurements. Error bars represent 1 S.D. The experiment was repeated twice. * $p < 0.001$

Table 1.

Proteins correlating with human plasma antioxidant activity and their abundances.

Protein ^I	Concentration	Reference
Peak 1		
Fibrinogen	1.7–4.1 mg/ml	(28)
α-2-macroglobulin	2.3–3.0 mg/ml	(29)
Peak 2		
Immunoglobulin G	7.1 – 18.5 mg/ml	(30)
Inter α. trypsin inhibitor 4	0.4 – 0.5 mg/ml	(31)
Apolipoprotein A-II	0.3 – 0.4 mg/ml	(32)
Serum amyloid P	0.03 mg/ml	(33)
Peak 3		
Albumin	38–50 mg/ml	(34)
Serotransferrin	2.7–4.4 mg/ml	(35)
α-1-antitrypsin	1.0–2.0 mg/ml	(36)

^I All proteins previously identified as correlating strongly ($r > 0.90$) with LDL antioxidation activity in human plasma (19).

Table 2.

Approximate Contributions of Each Peak and Identified Components (from AAPH experiments).

Location	% Plasma Activity	Identified Component(s)	% Plasma Activity
Peak 1	~15	Fibrinogen	~15
Peak 2	~25	Immunoglobulin G (80% of peak 2)	~20
		HDL (~5% of peak 2)	~1–2
		Other (~15% of peak 2)	~3
Peak 3	~40	Albumin	~40
Peak 4	~6	Small molecules (uric acid, etc.)	~6
Activity outside of ID'd peaks	~14	Unknown	~14
Total:	100		100