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ARTICLE *in* BIOCHEMISTRY · DECEMBER 2006

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Identification of Alpha-1 Acid Glycoprotein as a Lysophospholipid Binding Protein: A Complementary Role to Albumin in the Scavenging of Lysophosphatidylcholine[†]

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Received August 15, 2006; Revised Manuscript Received October 2, 2006

ABSTRACT: Alpha-1 acid glycoprotein (AGP, orosomucoid), a major acute phase protein in plasma, displays potent cytoprotective and anti-inflammatory activities whose molecular mechanisms are largely unknown. Because AGP binds various exogenous drugs, we have searched for endogenous ligands for AGP. We found that AGP binds lysophospholipids in a manner discernible from albumin in several ways. First, mass spectrometric analyses showed that AGP isolated from plasma and serum contained lysophosphatidylcholine (LPC) enriched in mono and polysaturated acyl chains, whereas albumin contained mostly saturated LPC. Second, AGP bound LPC in a 1:1 molar ratio and with a higher affinity than free fatty acids, whereas albumin bound LPC in a 3:1 ratio but with a lower affinity than that of free fatty acids. Consequently, free fatty acids displaced LPC more avidly from albumin than from AGP. Competitive ligand displacement indicated the highest affinity for AGP to LPC20:4, 18:3, 18:1, and 16:0 (150−180 nM), lysophosphatidylserine (K_d 190 nM), and platelet activating factor (PAF) (K_d 235 nM). The high affinity of AGP to LPC in equilibrium was verified by stopped-flow kinetics, which implicated slow dissociation after fast initial binding, being consistent with an induced-fit mechanism. AGP also bound pyrene-labeled phospholipids directly from vesicles and more efficiently than albumin. AGP prevented LPC-induced priming and PAF-induced activation of human granulocytes, thus indicating scavenging of the cellular effects of the lipid ligands. The results suggest that AGP complements albumin as a lysophospholipid scavenging protein, particularly in inflammatory conditions when the capacity of albumin to sequester LPC becomes impaired.

Alpha-1-acid glycoprotein (AGP[1]) is a 41-kDa acute phase protein, whose concentration in plasma increases from about 0.5 up to 3−4 g/L under inflammatory conditions. AGP carries five highly sialylated N-linked glycan chains, which, depending on the pathophysiological state, differ in their degree of branching and terminal carbohydrate residues (*1*). Although AGP is mainly synthesized by the liver, extrahepatic synthesis has been reported for endothelial cells and neutrophils (*2*, *3*). Freshly isolated human leukocytes contain AGP at their surface, and AGP accumulates at sites of inflammation (*3*, *4*). AGP binds with a nanomolar affinity to the CCR5 chemokine receptor of macrophages and to low affinity binding sites, which interact at least partially with the glycan chains of AGP (*5*). Furthermore, AGP binds to phospholipid vesicles and undergoes a pH-induced conformational change at the vesicle surface, which has been suggested to indicate the facilitated entry of AGP-bound drugs into cells (*6*).

AGP exerts a number of anti-inflammatory activities, whose molecular mechanisms have not been fully solved. It inhibits platelet aggregation and prevents superoxide production and chemotactic migration of neutrophils (*7*, *8*). AGP protects mice against tumor necrosis factor-α (TNF-α)-induced lethality and inhibits TNF-α-induced but not anti-Fas-induced apoptosis of hepatocytes in mice (*9*). It also protects against septic and hemorrhagic/hypovolemic shock in various animal models (*10*, *11*). These effects were attributed to the prevention of capillary leakage and maintenance of the capillary permeability barrier by AGP.

[†] This study was supported by the Academy of Finland (to P.S.).

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[1] Abbreviations:AGP, alpha 1-acid glycoprotein; ANS, 6-anilinonaphthalene-2-sulfonic acid; CE, cholesteroylester; ESI-MS, electrospray mass-spectrometry; FFA, free fatty acids; GalCer, galactosylceramide; GlcCer, glucosylceramide; HDL, high density lipoprotein; LacCer, lactosylceramide; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PAF, platelet activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SM, sphingomyelin; VLDL, very low density lipoprotein.

10.1021/bi061657l CCC: $33.50
Published on Web 11/08/2006

Interaction with lipopolysaccharide may contribute to the protective effect of AGP in sepsis (*12*). AGP also protects against ischemia/reperfusion injury by preventing apoptosis and inflammation (*13*, *14*). Surprisingly, transgenic mice, which constitutively overexpress AGP, are not protected against TNF-α-induced lethal shock and reperfusion injury, whereas exogenously administered AGP is protective (*14*, *15*).

AGP binds numerous basic and neutral lipophilic drugs as well as some acidic drugs and is a major drug carrier protein in plasma along with albumin (*16*). The primary binding site of AGP is formed by a fold and structural motifs characteristic for the lipocalin superfamily of proteins, which typically bind lipophilic ligands with a 1:1 stoichiometry (*17*, *18*). This suggests that the physiological function of AGP may be related to the binding of endogenous hydrophobic ligands. A few biological substances, particularly steroids (*19*), have been shown to bind to AGP, albeit with a relatively low affinity. Interestingly, AGP has been reported to bind platelet activating factor (PAF) and to modify the PAF-induced pyrogenic response *in vivo* (*20*, *21*). However, it has been argued that the binding of PAF does not explain the anti-inflammatory effects of AGP, and currently, it is unclear what the physiologically relevant ligands of AGP are (*22*).

To gain insight into the possible physiological ligands of AGP, we first searched for potential lipid ligands in purified AGP by electrospray mass-spectrometry (ESI-MS). We studied albumin in parallel with AGP because albumin is generally regarded as the major soluble lipid binding protein in plasma. We found significant amounts of lysophosphatidylcholine (LPC) in AGP isolated from plasma or serum. To further study the interaction of lysophospholipids with AGP, we investigated the binding of LPC and other lipids to AGP by ligand displacement and by stopped-flow kinetics. We also assessed the ability of AGP to transfer phospholipids between vesicles and scavenge their biological effects.

MATERIALS AND METHODS

Reagents. 6-Anilinonaphthalene-2-sulfonic acid (ANS) and luminol were purchased from Fluka (Buchs, Switzerland) and H_2DCFDA from Molecular Probes (Leiden, Netherlands). The unlabeled lipids were purchased from Avanti (Alabaster, AL), except for 16:0PAF and 16:0lyso-PAF, which were provided by Biomol (Plymouth, PA). The pyrene-labeled phospholipids and *N*-trinitrophenyl-phosphatidylethanolamine (TNP-PE) were synthesized as previously described (*23*, *24*). Organic solvents (HPLC-grade) were from Merck (Darmstadt, Germany) and other reagents from Sigma-Aldrich (St. Louis, MO). PAF and lyso-PAF were from Biomol (Plymouth Meeting, PA).

Protein Purification. Albumin was obtained from the manufacturing of pharmaceutical human plasma albumin prior to the addition of caprylic acid and pasteurization (Finnish Red Cross Blood Service, Helsinki, Finland). AGP was purified from Cohn fraction V supernatant by anion exchange chromatography and ultrafiltration (*25*). Each purification batch originated from about 4500 blood donors (1050 kg plasma). AGP and albumin were also purified from CPD-plasma and serum taken at the same time point from a single donor. Serum was separated after incubation of whole blood for 30 min at 37 °C and further incubated for 1 and 24 h at 37 °C to increase the amount of lysolipids in serum (*26*). Serum lipoprotein deficient fraction was prepared by adding 1.25 g of KBr per 1 mL of serum and ultracentrifugation for 46 h at 100,000*g*, followed by dialysis of the bottom fraction ($d > 1.25$ g/mL) three times against a 50-fold volume of PBS. Albumin was purified by Cibacron Blue Sepharose FF (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions and AGP by immunoaffinity chromatography. Polyclonal rabbit anti-human AGP IgG (Dako, Glostrup, Denmark) was affinity purified with human AGP immobilized to NHS-Sepharose, eluted at pH 3.0 and coupled to a High-Trap NHS-Sepharose column (Amersham Biosciences). AGP was eluted from the immunoaffinity column with 0.2 M glycine-HCl and 0.5 M NaCl at pH 3.0 and immediately neutralized with 1 M Tris base. Between purification runs, lipid-poor AGP was run through the column to bind residual lipids, and the column was washed at low and high pH. Plasma phospholipid transfer protein (PLTP) was purified and analyzed as described (*27*).

Lipid Extraction. For quantification of the lipid species by ESI-MS, the samples were supplemented with a mixture of internal standards and extracted in silane-treated screw-cap tubes according to Folch et al. except that the solvent contained 0.1 M HCl (*28*). The extracts were evaporated under a nitrogen stream, dissolved in chloroform/methanol 1:2 (v/v) and transferred into silylated 1.5 mL vials (Waters, Alltech, Milford, MI) for storage at −20 °C. NH_4OH (1%) was added to the extracts just prior to analysis.

Mass Spectrometry. The identification and quantification of the lipid molecular species was carried out with a Micromass Quattro Micro triple quadrupole instrument (Manchester, U.K.). The crude lipid extracts in 1:2 chloroform/methanol were infused into the source at a flow rate of 6 μL/min. Nitrogen was used as the nebulizer (500 L/h at 130 °C) and cone gas (50 L/h). The source temperature was set at 90 °C, and the potentials of the cone, extractor, and RF lens were 40, 2, and 0.3 V, respectively. The capillary voltage was 3.8 kV. The different phospholipid classes were selectively detected by using head group-specific precursor ion or neutral-loss scanning. LPC, phosphatidylcholine (PC), and sphingomyelin (SM) were detected by scanning for the precursors of 184 in the positive ion mode, phosphatidylethanolamine (PE) and phosphatidylserine (PS) by scanning for the neutral loss of 141 and 185, respectively, in the positive ion mode, whereas LPA was detected by scanning for the precursors of 153 in the negative ion mode (*29*). The collision energy was set to 20−55 eV, and argon was used as the collision gas. The spectra were smoothed and transferred to Microsoft Excel, and the relevant peaks were extracted using a Visual Basic macro. Quantification was carried out as described (*30*).

Analysis of Lysolipid Binding Stoichiometry. AGP and albumin at a concentration of 100 μM were incubated with different concentrations of lysophospholipids for 30 min in 10 mM Tris HCl and 150 mM NaCl at pH 7.4 (TBS). The protein and free lipids were separated in a Superdex 75HR gel filtration column (Amersham Biotech) with a flow of 0.5 mL/min. The effect of free fatty acid (FFA) on binding was studied by adding 700 μM oleic acid to the incubation in silylated vials. Protein content of the fractions was determined by absorbance at 280 nm using extinction

coefficients of 0.530 and 0.893 L g^{-1} cm^{-1} for albumin and AGP, respectively. Phospholipids were determined by phosphate analysis (*31*).

Neutrophil Activation Assay. Human polymorphonuclear leukocytes (PMN) were isolated from peripheral blood by dextran sedimentation and density centrifugation on Ficoll-Paque (Amersham Healthcare). The remaining erythrocytes were removed by hypotonic lysis. PMNs were suspended in Hank's balanced salt solution buffered with 10 mM HEPES at pH 7.4 (HBSS), and superoxide generation was monitored in 200 μL of HBSS containing 2×10^6 PMNs, 63 μM luminol, and the activators tested. Stock solutions of the lipids studied were prepared in 10 mM HEPES at pH 7.4, with tip sonication. Chemiluminescence was monitored in Opti-Plate F96 wells at 37 °C in a Victor II 1420 multilabel counter (Wallac Perkin-Elmer, Turku, Finland). For the measurement of intracellular reactive oxygen species (ROS), PMNs (2×10^6/mL) were loaded with 10 μM H_2DCFDA in HBSS for 30 min at 37 °C, the cells were washed and suspended into HBSS (5×10^6 cells/mL), and ROS generation was monitored by fluorescence measured at 2 min intervals using 485 nm for excitation and 535 nm for emission in the Victor II 1420 multilabel counter.

ANS Competition Studies. ANS was first titrated with the proteins studied. The measurements were carried out on a Varian Cary Eclipse fluorescence spectrophotometer at 20 °C. The excitation and emission wavelengths were 340 and 430 nm and bandwidths of 5 and 10 nm, respectively. The K_d values were determined from binding isotherms by fitting the 1:1 interaction formulation or competitive equations to the data points. The user-defined quadratic fitting equation to derive the apparent 1:1 dissociation constant K_d for ANS was $Q = c/2 + z/2 - 1/2\{(c + z)^2 - 4z\}^{1/2}$ ⟨1⟩, where $c = 1 + K_d/[\mathrm{ANS}]_{tot}$, and z was the concentration of AGP or albumin (Sigma Plot 2000; Aspire Software International, Leesburg, VA).

The displacement data were normalized between 0 and 1 and fitted using the equation $Q = [r]/(\{K_r(1 + [\mathrm{I}]/K_I)\} + [r])$ ⟨2⟩, where variable r is the concentration of the competitor, [I] the constant concentration of inhibiting ANS, K_I the dissociation constant of ANS, and K_r the dissociation constant of the competitor. The data were fitted by keeping either both of the dissociation constants free or by measuring the K_r by using the known affinity to inhibitor measured separately in a two-reagent titration. Cooperative binding isotherms were fitted using the Hill's equation with four parameters, $y = y_0 + ax^b/c^b + x^b$ ⟨3⟩.

Fluorescent Phospholipid Binding and Transfer Assays. Uptake of phospholipids from vesicles was studied using vesicles containing pyrenylacyl PC, SM, PE, PS, PG, cholesteryl ester, or ceramide and TNP-PE, a quencher of pyrene fluorescence. The increase of the pyrene monomer fluorescence upon the addition of aliquots of the protein to the quenched donor vesicles was measured. In transfer measurements, unquenched vesicles were also employed (*32*). The excitation and emission wavelengths were 345 and 376 nm, and the bandwidths 5 and 10 nm, respectively. The donor vesicles consisted of 1-palmitoyl-2-oleyl-phosphatidylcholine (POPC), TNP-PE, and a pyrene-labeled phospholipid in a molar ratio of 89:10:1 and acceptor vesicles of POPC and 1-palmitoyl-2-oleyl-phosphatidic acid (POPA) (9:1) in TBS. The lipids were mixed in chloroform/methanol (9:1, by vol), dried under a nitrogen stream, and then kept under high vacuum for 1 h to remove any residual solvents. One milliliter of TBS buffer was added, and the lipids were dispersed by probe sonication three times for 2 min at 20 °C in a water bath to yield small unilamellar vesicles. Any undispersed lipid and titanium particles were removed by brief centrifugation.

Stopped-Flow Measurements. The association of LPC18:1 and FFA20:4 with AGP or albumin were monitored by altered Trp fluorescense intensity using an MOS250 fluorometer coupled to a μSFM-20 stopped-flow apparatus equipped with a z-shaped flow-through cuvette (10 × 1 mm, BioLogic, France). First-order rate constants were obtained by fitting the experimental data to single exponentials using Biokine software and the simplex method. The change in Trp intensity was high enough only in the case of the LPC/AGP interaction (30% enhancement after 40 ms dead time). The on-rates were well described by a single-exponential function over the entire range of LPC/AGP molar ratios from 1:1 to 25:1. The excitation and emission wavelenghts were 290 nm 335 nm, respectively. At least 10 time scans with about 1000 data points for each reaction were acquired.

RESULTS

Identification of the Endogenous Lipids in AGP and Albumin. We first studied whether AGP and albumin purified from a large plasma pool originating from several thousand donors contained phospholipids. Lipids were extracted from the purified proteins and then subjected to analysis by ESI-MS. The most abundant phospholipid found both in AGP and albumin was LPC. AGP contained 3.2 mol % and albumin 2.1 mol % of LPC (Figure 1A and B). AGP also contained significant amount of PC and LPA, whereas only LPA was detected in albumin. Only trace amounts of other lipids (SM, PA, S1P, SPC, PE, and PS) were detected.

To further compare the amount of LPC in AGP and albumin, the proteins were purified from the same lipoprotein-deficient plasma and serum samples by affinity chromatography. The different AGP preparations contained 3−10 mol % LPC (Figure 1). The level of LPC was somewhat lower in the AGP purified by large scale fractionation from a plasma pool than that in the AGP isolated by affinity chromatography from single serum and plasma samples, which may be due to the dissociating effect of the acid ethanol precipitations during the 1000 L volume fractionation. The albumin preparations contained 2−5 mol % of LPC. The proportion of PC was about 2 mol % in AGP and about 0.3 mol % in albumin. Other lipids were present only in trace amounts.

The dominating acyl chain in LPC in raw plasma and serum and in the purified albumin preparations was 16:0, whereas in purified AGP, 18:1, 18:2, and 20:4 acyl chains were more prevalent (Figure 2). This prevalence of mono and polyunsarurated acyl chains in AGP-associated LPC was most prominent in serum samples, both in freshly isolated serum and serum incubated for 24 h.

Stoichiometry and Affinity of Lysophospholipid Binding. To study the lysophospholipid binding capacity of AGP and albumin, the proteins were incubated with various lysolipid species at different molar ratios and separated from free lipids by size exclusion chromatography. AGP bound LPC18:1 up

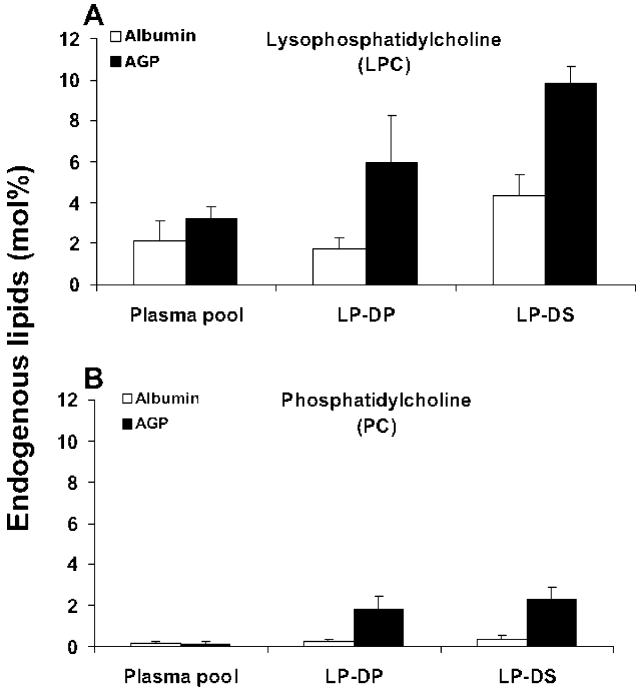

FIGURE 1: Identification and quantification of endogenous lipid ligands in AGP and albumin. The amount of LPC (A) and PC (B) in AGP (filled columns) and albumin (open columns) purified by cold ethanol fractionation from pooled human plasma (plasma pool) or by affinity chromatography from lipoprotein-deficient plasma (LP-DP) or serum (LP-DS) of a single donor. The lipids were quantitated by triple quadrupole ESI-MS/MS after extraction from the proteins. The results are represented as means ± S.D. of three independent assays.

to a molar ratio of 1:1 (Figure 3). Albumin bound lysolipids with a molar ratio close to 3:1. When oleic acid was present in molar excess, LPC was displaced more effectively from albumin than from AGP (Figure 3).

We next studied the affinity of various lipids for AGP and albumin by measuring their ability to displace the fluoroprobe ANS from these proteins. For comparison, the affinity for several steroids was also measured. Albumin has several binding sites for ANS, whereas AGP has only one (*33*). Thus, in the case of albumin, the K_d values are only apparent, that is, averages of those for the different binding sites.

The affinity of different LPC species to AGP was higher than that of free fatty acids (Table 1). The affinity of the different lysophospholipids to AGP varied depending on the polar head group and saturation as well as the length of the fatty acyl chains (Table 1). The lowest K_d values were measured for LPC20:4, 18:3, 18:1, and 16:0 (143−179 nM) and LPS18:1 (192 nM). We also studied the much less abundant bioactive phospholipid PAF (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine), which displayed an affinity approximately 10-fold higher (K_d 235 nM) than that previously described (*20*). Notably, AGP bound steroids with clearly lower affinity (K_d 1−3 μM) (Table 1, Figure 4). Binding of intact phospholipids could not be measured reliably because of their very low critical micelle concentration.

Albumin displayed a lower affinity than AGP for most of the ligands (Table 1). The apparent K_d of free fatty acids to albumin, as determined by the ANS competition assay, was lower than that of any lysophospholipid, which was consistent with the efficient displacement of LPC from albumin by free fatty acids. Albumin displayed sigmoidal binding isotherms, especially for fatty acids (Figure 4), thus implying positive cooperativity between the different binding sites. This is consistent with the structural data implying drastic conformational changes induced by fatty acids in the three lobes of the all-alpha fold of albumin (*34*). Alternatively, it is possible that ANS does not bind to the highest affinity sites of albumin primarily occupied by fatty acids, which could explain the sigmoidal binding curve. In contrast, AGP displayed simple hyperbolic binding curves for the high affinity lysolipid ligands (Figure 4). The affinity of the lysophospholipids to AGP was clearly higher than that to the apparent affinity values to albumin (Figure 4).

Kinetics of Lysophospholipid Binding to AGP. To study the association and dissociation rates of lipids with AGP, we monitored the Trp fluorescence of AGP by stopped-flow kinetics. The observed association rates (k_{obs}) of LPC18:1 and AGP revealed strict dose dependence. The results with optimal signal (final concentration of 2.5 μM AGP) are plotted in Figure 5A. The on-rate as judged from the slope was 6.0 μM^{-1} s^{-1} (assuming a 1:1 stoichiometry), and the off-rate as extrapolated from the *y*-axis intercept was 25.3 s^{-1}. The dissociation rate from AGP was faster for FFA20:4 and slower for LPC18:1, following both the association rate of excess ANS (rate-limiting ligand dissociation; 5.9 ± 1.6 s^{-1} and 1.7 ± 0.6 s^{-1}, respectively) and the dilution equilibration (8.6 ± 0.4 s^{-1} and 0.9 ± 0.3 s^{-1}, respectively). The 3.0- to 9.3-fold slower dissociation and the higher affinity of LPC than FFA correspond within reasonable limits to the steady-state kinetics (equilibrium) results from the ANS displacement experiments. Because the measured dissociation rate of LPC was on average 21-fold slower than that deduced from the association plot, this implied an induced-fit mechanism in the binding of LPC by AGP. The K_d value (k_{off}/k_{on}) for AGP−LPC18:1 was 4.2 μM when extrapolated from the on-rate plot, whereas the measured value was 154−283 nM, thus independently confirming the K_d from ANS competition (166 nM).

Binding and Transfer of Pyrene-Labeled Lipids from Vesicles. AGP was able to bind all pyrene-labeled phospholipid species studied from both small and large unilamellar donor vesicles, albeit with varying efficacy (Figure 6). Phospholipids with short fatty acyl chains and lysophospholipids were more efficiently taken up by AGP than phospholipids with long acyl chains. The pyrene phospholipids bound to AGP, from both small and large planar vesicles, in the following order: LPC > LPA > PS > SM > PC > PA. The glycosphingolipids glucosylceramide, galactosylceramide, and lactosylceramide were less avidly bound by AGP. Albumin was clearly less effective than AGP in sequestering intact lipids form vesicles. Among phospholipids, albumin took up only PS and SM (Figure 6 F−H) and more avidly bound lysophospholipids. It was not feasible to calculate the absolute K_d values because of the fact that the affinities of the different lipids for the donor vesicle membrane vary greatly (*24*, *32*).

We also tested whether AGP or albumin could stimulate the efficient transfer of pyrene-labeled phospholipids from small (25 nm) unilamellar vesicles to HDL particles. However, neither protein enhanced the transfer of pyrene-labeled PC, SM, PE, PS, or PG from their basal rate under conditions where efficient translocation by purified phos-

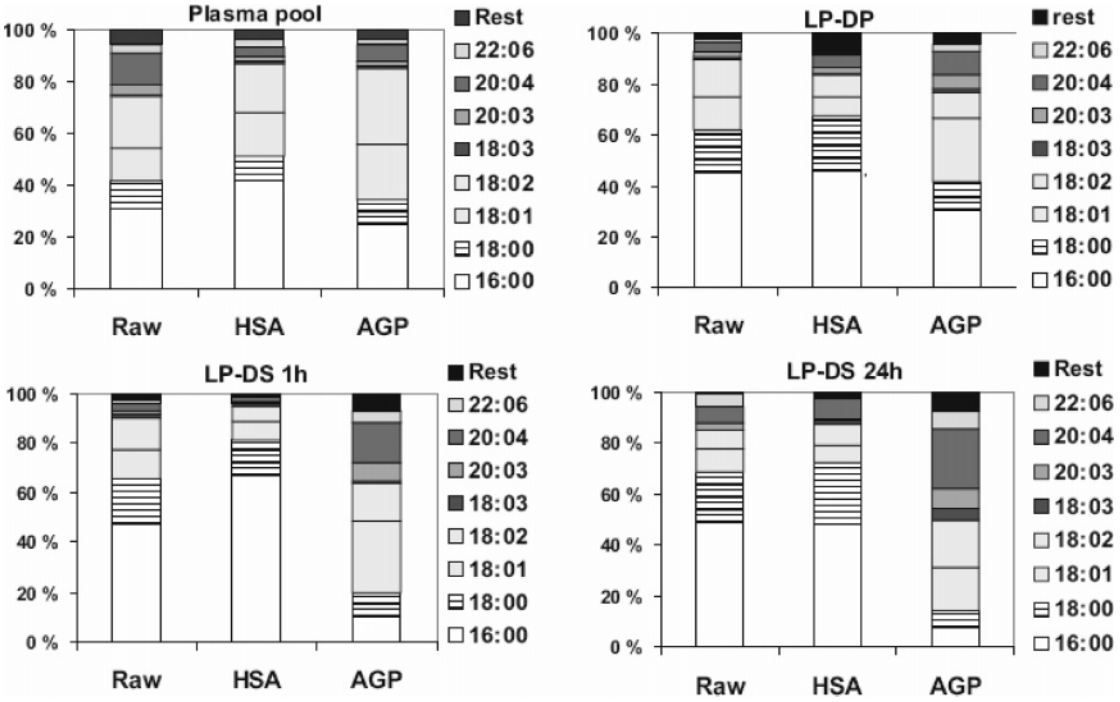

FIGURE 2: Molecular species of LPC in purified AGP and albumin and in the source plasma or serum. AGP and albumin were purified as in Figure 1 and additionally from serum, which had been incubated for 24 h (LP-DS 24 h) before the removal of lipoproteins and purification of the proteins. The molecular species of LPC were analyzed by ESI-MS. The results are presented as percentages of total fatty acyl substitution species determined, the means of three analyses.

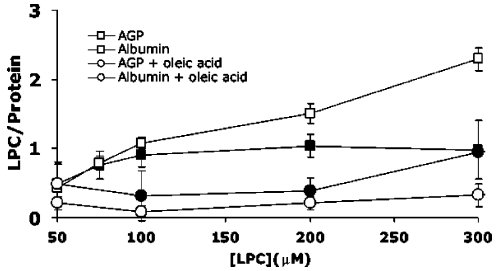

FIGURE 3: Binding of LPC to AGP and albumin and competition by free fatty acids. AGP and albumin at 100 μM were incubated with different concentrations of LPC for 30 min at 20 °C followed by separation of the protein and free lipid by size exclusion chromatography. The effect of free fatty acid (FFA) on binding was studied in the presence of 700 μM oleic acid. Protein was determined by absorbance at 280 nm using specific molar extinction coefficients and phospholipids by the method of Bartlett (*30*). (■) AGP; (●) AGP incubated with 700 μM oleic acid; (□) albumin; (○) albumin incubated with 700 μM oleic acid. The results are the means ± S.D.; $n = 3$.

pholipid transfer proteins, that is, PC-TP, nsLTP, and plasma PLTP, was observed (results not shown). Thus, AGP does not seem to act as a lipid transfer protein.

Effect of AGP on Phospholipid-Mediated Granulocyte Activation. To study the influence of AGP on the cellular effects of phospholipids, we monitored the intracellular generation of reactive oxygen species (ROS) in PMNs by an H_2DCFDA probe. PAF caused a dose-dependent increase in ROS generation, which was effectively prevented by AGP (Figure 7A). AGP alone had little effect on intracellular ROS generation, whereas albumin inhibited it. Albumin also prevented the ROS generation induced by PAF. The intracellular ROS generation was prevented by DPI, an inhibitor of NADPH oxidase. In line with earlier reports (*8*), we observed a dose-dependent inhibition of fMLP-induced extracellular superoxide generation by AGP (Figure 7B).

Table 1: Parameters for the Binding of Different Lipids to AGP and Albumin and the Cooperative Interaction of Albumin with its Ligands[a]

	AGP			Albumin			
ligand	K_d (nM)	r^2	S.E.M. of the fit	apparent K_d (nM)	r^2	S.E.M. of the fit	Hill's constant
LPC20:4	143	0.96	0.049	969	0.93	0.049	1.48
LPC18:3	151	0.97	0.045	763	0.95	0.035	1.53
LPC18:1	166	0.99	0.032	764	0.95	0.052	1.14
LPC16:0	179	0.98	0.039	851	0.93	0.059	1.44
LPS18:1	192	0.91	0.099	634	0.88	0.098	2.22
PAF16:0	235	0.98	0.038	1693	0.97	0.021	1.91
LPG18:1	266	0.94	0.086	638	0.88	0.095	1.74
FFA22:6	348	0.98	0.032	833	0.96	0.050	2.24
LPE18:1	397	0.99	0.028	1235	0.91	0.058	1.95
FFA18:1	404	0.97	0.053	476	0.82	0.147	1.99
lysoPAF16:0	441	0.96	0.048	1648	0.95	0.026	1.90
LPA18:1	530	0.92	0.098	606	0.84	0.128	2.04
FFA20:4	717	0.97	0.054	1001	0.93	0.085	2.12
LPI-mixture	962	0.84	0.118	1036	0.88	0.073	1.47
SPC18:1	1804	0.84	0.025	1416	0.95	0.025	1.52
PC16:0;18:1	2072	0.90	0.050	1636	0.96	0.026	1.11
progesterone	1173	0.86	0.043	1769	0.98	0.024	2.12
S1P18:1	2205	0.92	0.046	1285	0.91	0.054	1.30
testosterone	2936	0.54	0.051	2661	0.74	0.043	0.85
ANS	1638	0.92	0.091	848	0.98	0.041	

[a] The ligands are tabulated in descending order in terms of affinity to AGP. Most pronounced differences in the binding to AGP and albumin were observed for PAF, lysoPAF, and lysoPE. The measurements were carried out as explained in Figure 4.

Again as previously described (*35*), LPC potentiated superoxide generation by fMPL, which was mainly seen in the slower second phase of the biphasic response. AGP prevented this potentiation when present at a molar ratio of 1:1 or higher. Albumin had no effect on fMLP-induced superoxide generation but prevented LPC-induced potentiation.

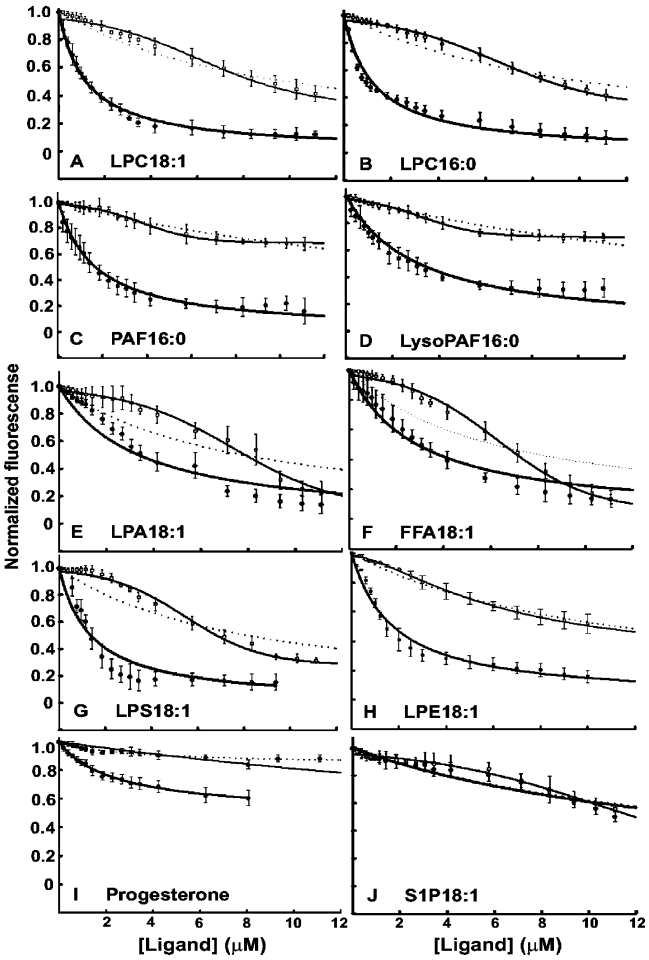

FIGURE 4: Affinity of AGP and albumin for various lipids measured by displacement of ANS. The results represented are plotted as a function of increasing amounts of competitor in the presence of a constant amount of protein and ANS (1 μM and 10 μM, respectively), in order to obtain adequate increase of fluorescence and to allow the approximation $[\text{protein}]_{\text{free}} = 0$. (●) AGP (lower traces); (□) albumin (upper traces). Thick solid lines show the 1:1 stoichiometric fits for AGP; thin solid lines show the sigmoidal fits for albumin; and dotted lines show the fits indicating the apparent K_d for albumin. Only sn-1-isoforms of lysophospholipids were available for testing. The results are the means ± S.D.; $n = 3$. The measured dissociation constant of ANS to albumin was 850 nM and to AGP was 1640 nM and were used in the fitting equation when calculating the K_d values for the different lipids. Final ethanol concentration was <1%.

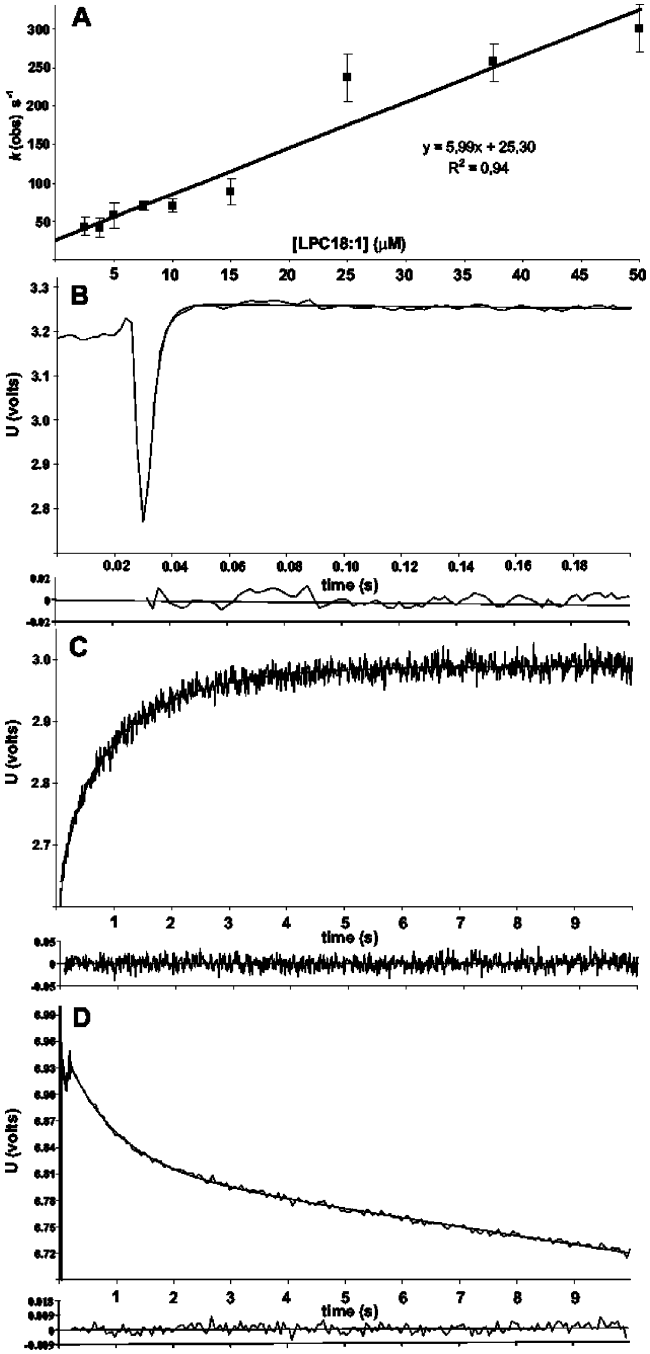

FIGURE 5: Kinetics of the AGP-lipid interaction. (A) On-rates and extrapolated off-rates for AGP-LPC18:1 association (2.5 μM AGP titrated by increasing concentrations of LPC18:1). (B) Example trace of the association data from the fastest data point (2.5 μM AGP with 50 μM LPC18:1). (C) First order off-rate from AGP, as followed by the association of the excess ANS probe (100 μM) to 2.5 μM AGP-LPC18:1 complex or (D) the direct dissociation of 10 μM AGP-LPC18:1 complex after a 1:5 dilution. The time course for dissociation could be fitted to a single exponential, although two components whose rates differ by less than 2-fold cannot be resolved from the stopped-flow data. Residuals are shown in a separate panel below the corresponding Figures. The results are the means ± S.D.; $n = 3$.

DISCUSSION

AGP is known to bind numerous hydrophobic or cationic drugs, albeit with a relatively low, micromolar affinity (*16*). We have searched for endogenous molecules that could explain at least part of the potent anti-inflammatory and cytoprotective effects of AGP. We first analyzed lipids in AGP preparations isolated from human plasma and serum and then characterized the interactions of the identified lipids with AGP. The mass spectrometric method used in our study can identify hundreds of lipid species with high accuracy and sensitivity (*36*) and allowed us to unambiguously identify protein-bound phospholipids, their acyl chain length, and the number of double bonds. The most prevalent lipid ligand in AGP proved to be LPC with predominantly mono and polysaturated acyl chains. In accordance with the findings of the mass spectrometric studies, LPC displayed the highest affinity to AGP of the various lysophospholipids, fatty acids, and lysosphingolipids tested.

LPC is formed from membrane phospholipids by the secretory phospholipase A2 type IIa, a known marker of inflammation, which hydrolyzes the sn2 ester bond of phospholipids to produce free fatty acids and LPC (*37*). Up

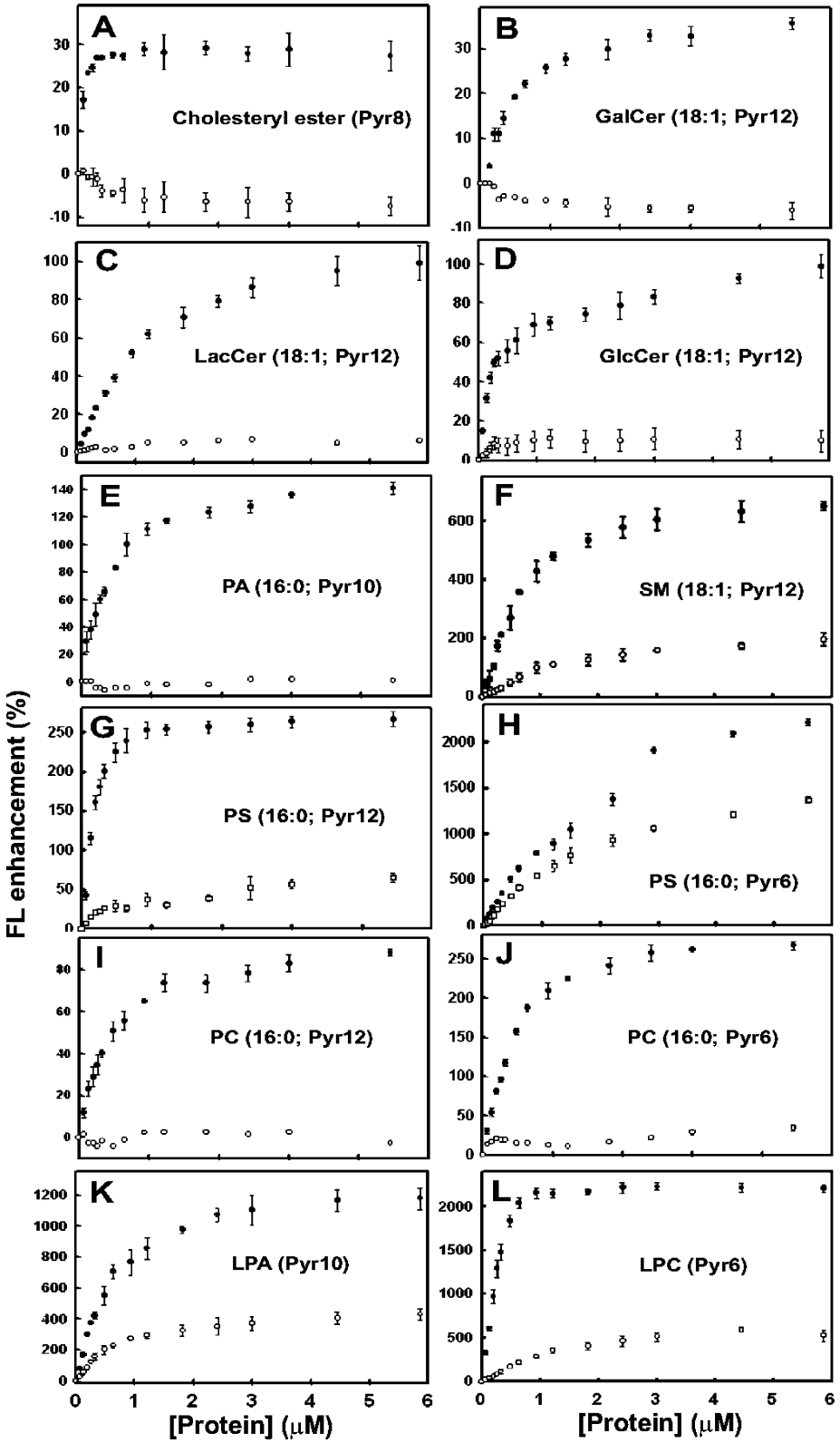

FIGURE 6: Uptake of pyrene-labeled lipids from vesicles by AGP and albumin. The uptake of different lipids was studied using vesicles containing sn2-pyrenylacyl-substituted lipids and TNP-PE, a quencher of pyrene fluorescence. The increase of the pyrene monomer fluorescence upon the addition of aliquots of the protein to the quenched donor vesicles is a measure of uptake by the protein. (●) AGP; (○) albumin. Please note the different scales on the *y*-axis. The results are the means ± S.D.; $n = 3$.

to 80% of LPC in plasma is found in the non-lipoprotein fraction, where albumin is considered as the main lipid binding protein. Unsaturated LPCs prefer the albumin fraction over the monolayered lipoproteins, and physiologically, albumin is thought to carry as much LPC as FFA (*38*). However, only about half of the 18:2-LPC and one-third of the 20:4-LPC have been reported to exist as albumin-bound forms (*39*), which indicates the existence of other LPC-binding proteins in plasma.

There is accumulating evidence on potent biological activities of LPC. It has been shown to be released from apoptotic cells after caspase-3 mediated activation of the

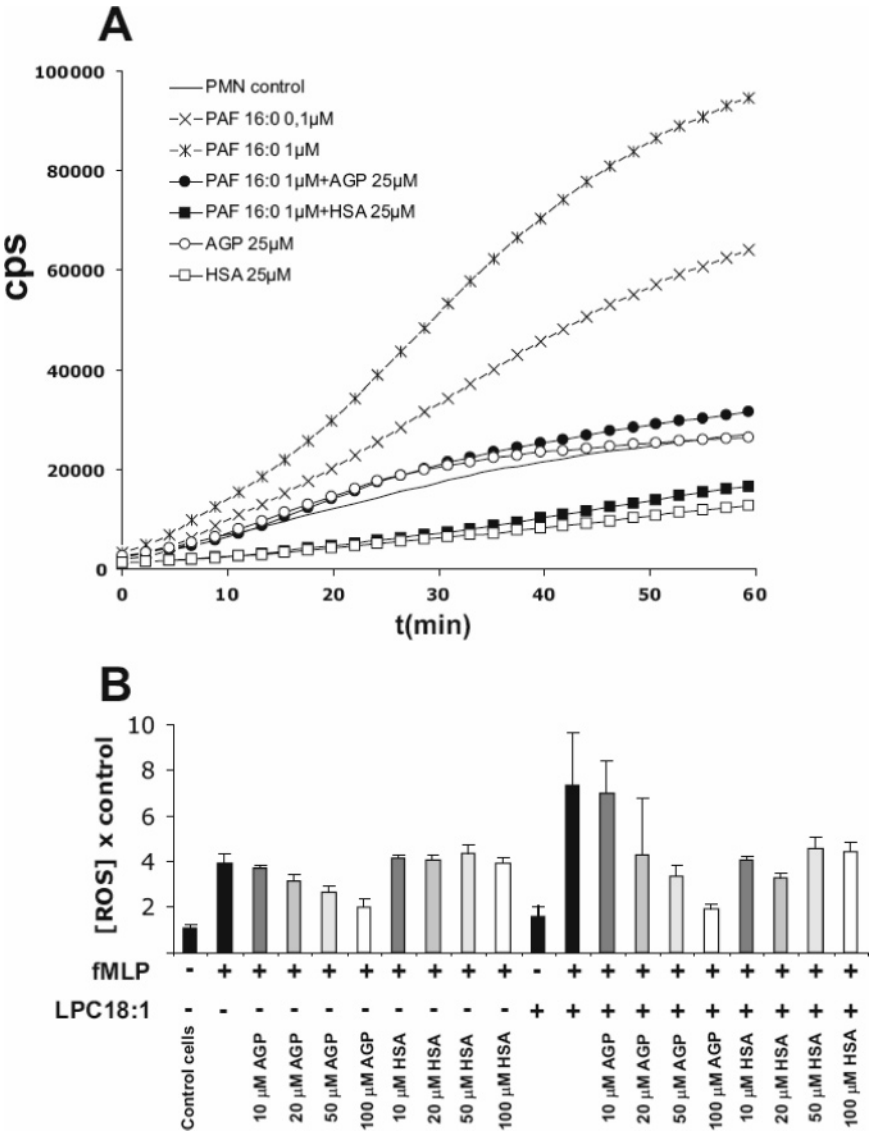

FIGURE 7: Inhibition of neutrophil activation by AGP and albumin. (A) Accumulative kinetic traces from PAF-induced intracellular ROS generation monitored by DCF fluorescence. PMN density was 10^6 mL^{-1}. The results are the means of three experiments ± S.D. (B) Real time superoxide generation induced by 1 μM fMLP and 20 μM LPC18:1. Superoxide generation was monitored by luminol. PMN density was 10^7 mL^{-1}. The results show the area under the curve and the means ± S.D.; $n = 6$.

calcium-independent PLA2 and to induce the chemotactic migration of monocytes (*40*). The chemotactic effect of LPC was mediated through Gi-independent G2A GPCR signalling (*41*). In another line of research, LPC has been shown to increase endothelial permeability and contribute to the loss of vascular integrity in inflammatory conditions, acting via GPR4 (*42*, *43*, *44*). LPC has also been found to produce an ischemia-like effect in perfused working rat hearts, including Ca2+-overload (*45*). Although the existence of G-protein-coupled receptors for bioactive lipids PAF, LPA, S1P, and SPC is well established, the specificity of the suggested LPC receptors G2A and GPR4 has been controversial because of the retraction of the original papers that reported their 30 nM and 160 nM affinity to LPC (*46*). The cellular effects of free LPC might be caused by detergent-like effects, but cytotoxicity is only seen above ~30 μM (*47*).

Because the administration of AGP has been shown to maintain endothelial integrity in experimental inflammatory conditions (*10*), it is possible that this protective effect is at least in part mediated via the scavenging of LPC. Furthermore, AGP also bound with high-affinity PAF, which already increases endothelial permeability at nanomolar concentrations (*48*). Interestingly, it was recently shown that AGP modulated the pyrogenic activity of infused PAF *in vivo* (*21*). The earlier notion that the cytoprotective effects of AGP *in vivo* would not be mediated by the binding of PAF was based on experiments in which PAF was administered to mice in amounts clearly exceeding the 1:1 molar ratio to the co-administered AGP (*9*). It is, thus, possible that the protective effect of AGP on endothelial integrity is also mediated by the scavenging of PAF, aside the over 1000-fold more abundant LPC.

LPC is present at much higher concentrations in plasma (about 100−250 μM) than the other lysolipids (*26*, *49*). LPC is generally thought to be buffered by albumin so that the concentration of free LPC remains very low. Accordingly, it was demonstrated that albumin prevented LPC-induced permeability enhancement of vascular endothelium when the molar ratio of LPC to albumin was three or less (*50*). This is in line with the stoichiometry that was found for the LPC/albumin complex in the present study. Although in normal conditions albumin's concentration is about 50-fold higher

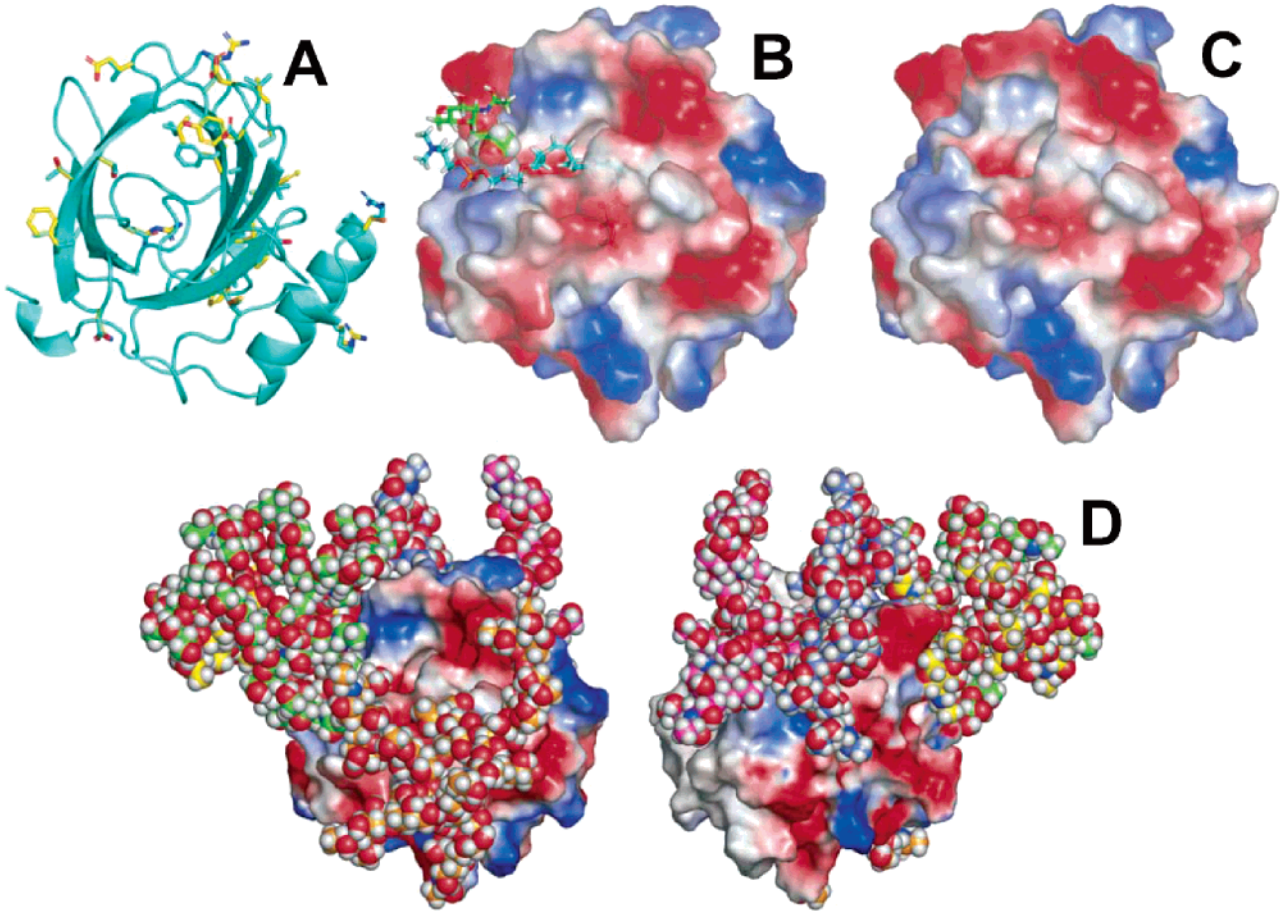

FIGURE 8: AGP models. (A) Comparison of AGP-1 and AGP-2 with the differing 21 residues illustrated. AGP-1, carbon atoms in cyan; AGP-2, carbon atoms in yellow. (B) Possible mode of binding of LPC20:4 to AGP-1. Carbon atoms in cyan: the lysolipid ligand; carbon atoms in green: the first two residues of the glycan chain attached at N75, first residue in space-filling spheres and the second in sticks. Protein surface colored according to electrostatic potential. (C) Electrostatic potential of a model of AGP-2. (D) Position and size of N-linked oligosaccharides. The carbon atoms in the glycans are colored to distinguish the five chains: glycosylation site I at N15, pink; II at N38, orange; III at N54, yellow; IV at N75, green; and V at N85, lilac. (A) to (C) are all seen from the same direction and in same scale. In (D), the first part is from the same direction, and the second part is 180° vertically rotated.

than that of AGP, during severe inflammation and sepsis, the difference diminishes remarkably because the level of albumin decreases down to 50%, whereas that of AGP increases up to 5-fold, close to 100 μM. The finding that oleic acid effectively displaced LPC from albumin suggests that under the pathophysiological conditions associated with elevated free fatty acid level in circulation, the capacity of albumin to scavenge LPC may become limited. As the affinity of LPC to AGP was clearly higher than that of fatty acids and the competition of LPC binding to AGP by oleic acid was less efficient than that to albumin, AGP's function as a LPC scavenger could be important in conditions associated with the elevated free fatty acid level. For instance, in the metabolic syndrome and diabetes, the molar ratio of FFA to albumin may be up to six (*51*), thus limiting the capacity of albumin to scavenge LPC.

There are a few other clues as to how AGP might complement albumin as a lysophospholipid scavenger in inflammatory conditions. The prevalence of mono- and polyunsaturated LPC in purified AGP and the ability of AGP to pick up lysolipids directly from phospholipid membranes suggest that AGP may sequester LPC in proximity to its generation in the plasma membrane. The previous findings of the ability of AGP to bind to homogeneous phospholipid vesicles and undergo a pH-induced conformational change led to the concept of a facilitated release of AGP-bound drugs into the cells (*6*). Considering the sequestering of LPC, the direction of AGP-assisted ligand flow in the membrane interface might also be the opposite. The stopped-flow kinetics data suggesting a low off-rate and an induced fit in LPC binding to AGP indicates a safety-catch mechanism in the sequestering of LPC. Albumin, instead, is thought to primarily bind free fatty acids and LPA, whereas LPC binds in a different mode and with lower affinity (*52*). Furthermore, the ability of AGP to recognize specific cell surface molecules, such as the CCR5 receptor and lectins (*53*), and become associated with the cell surface (*4*) indicates the ability of AGP to become enriched at sites of LPC generation.

The binding of long and bulky drugs induce a larger conformational change in AGP upon binding (*54*), in accord with the changes in other lipocalin structures upon ligand binding (e.g., retinol binding protein 1KT5 vs 1HBQ, beta lactoglobulin 1BEB vs 1GX9, or the artificial lipocalin diga16 1KXO vs 1LKE, in PDB). To characterize the high-affinity interaction of AGP with polyunsaturated LPC, we constructed a model of AGP on the basis of new sequence data of lipocalin family members, benefiting from the superimposing of known lipocalin structures to align the two outlier AGP sequences to kernel lipocalins. In the two

previous models (*55*, *56*) either the second or third N-glycosylation site is placed in the interior of the protein, although all five N-gycosylation sites of AGP are known to carry complex-type, sialylated glycans (*1*). Figure 8A highlights the sequence variations between the two main human plasma AGP isoforms, AGP-1 and AGP-2. Seven of the 21 differences are clustered at the entrance to the binding cavity. Another cluster of five differing residues is located under the α helix. Figure 8B and C shows the electrostatic surfaces of AGP-1 and AGP-2 models, respectively. The most prominent differences are in the distribution of charges at the mouth of the cavity. Figure 8B shows a typical docking result of LPC20:4 to the AGP-1 model. Repetitive docking experiments demonstrated that LPC20:4 adapts to the cavity of the lipocalin fold with the acyl chain inward and preferably in a bent conformation. The docking results did not conclusively show any preferred location of the phosphocholine moiety. Figure 8D illustrates the approximate scale of the glycan moieties relative to protein when sialylated complex-type glycan chains (48% by weight) were inserted into the five N-glycosylation sites using the reported branching. Because of the clustering of the glycosylation sites, more than 50% of the protein surface is available for protein−protein interactions. However, because of the large conformational freedom of the sugar chains, they are capable of interacting with a surface of the protein moiety larger than that shown in the static model. The glycan at N75 (green) has a potential to close the binding site, which is interesting in light of the kinetic induced fit observation. An animation of the AGP-1 model with LPC20:4 is provided in Supporting Information, together with all molecular coordinates and sequence alignments.

In conclusion, our results propose a new mechanism that helps to explain the cytoprotective properties of alpha-1 acid glycoprotein. AGP complements albumin as a LPC scavenger, particularly during inflammation when its concentration remarkably increases relative to that of albumin. A further property making AGP an efficient scavenger of LPC in inflammation is its ability (i) to bind phospholipids more effectively than albumin from vesicle surfaces; (ii) to prefer unsaturated LPC; (iii) to resist competition by free fatty acids; and (iv) to possess an induced-fit mechanism, which provides a slow off-rate for the sequestered lysophospholipids.

ACKNOWLEDGMENT

Modeling software, databases and support was provided by CSC - Scientific Computing, Finland. We are grateful to Tarja Grundström, Jukka Halme, Teija Kupari, Sisko Lehmonen, Jukka Lehtiniemi, and Jari Metso for technical assistance.

SUPPORTING INFORMATION AVAILABLE

Description of the methodology behind molecular modeling of the AGP proteins and its glycans, LPC-docking as well as the coordinates, multiple sequence alignment, and animation on the structural interface between AGP and LPC. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI061657L