Binding of Annexin V to Membrane Products of Lipid Peroxidation†

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ABSTRACT: There is increasing evidence that endogenously generated aldehydes formed as a result of lipid peroxidation are involved in the pathophysiological effects associated with oxidative stress in cells and tissues. Malondialdehyde (MDA), a major product of lipid peroxidation, can modify amines present on the cell surface and thereby introduce negative charges that can affect the interfacial ionic layer. We show that lipid peroxidation of RBC generates MDA adducts that, similar to phosphatidylserine (PS), bind annexin V in a Ca^{2+}-dependent manner. Like PS, these adducts also promote the "PS-dependent" prothrombinase assays, albeit to lower levels. These results indicate that annexin V binding cannot be used as an exclusive indicator of cell surface PS and raise the possibility that some phenomenon attributed to PS may, in fact, also involve aldehyde−lipid adducts.

Lipid peroxidation, the nonenzymatic autocatalytic interaction of polyunsaturated fatty acids (PUFA) with molecular oxygen, is a process common to all biological systems (*1*). There is increasing evidence that endogenously generated aldehydes formed as a result of lipid peroxidation are involved in the pathophysiological effects associated with oxidative stress in cells and tissues. Unlike free radicals, aldehydes are relatively long-lived and diffuse from their site of origin (the lipid bilayer) and react with multiple intra- and extracellular targets far distant from the initial free radical event (*1*). Indeed, there have been many reports associating increased levels of malondialdehyde (MDA)[1] with aging and senescence (*2*), myocardial infarction (*3, 4*), stroke (*5*), diabetes (*6*), cancer (*7*), and apoptosis (*8*).

Annexin V binds in a Ca^{2+}-dependent manner to PS and other negatively charged phospholipids in membranes (*9*). While the negative charges provided by the phospholipid headgroup participate in the binding of annexin V, other negatively charged moieties can substitute for phospholipid. For example, negatively charged amphiphiles, such as oleic acid or dodecyl sulfate, can substitute for PS and promote Ca^{2+}-dependent binding of annexin V (*10*). These data suggest that any intrinsic modification to cell membrane lipids that introduces negative surface charges at the interfacial ionic layer could promote annexin binding. Since lipid peroxidation generates amine-reactive aldehyde intermediates (*1*) that can react with aminolipids to produce a negatively charged moiety, we determined whether such aldehyde−lipid adducts bind annexin V. Our results demonstrate Ca^{2+}-dependent binding of annexin V to MDA−lipid adducts that is comparable to its binding to PS. Similar to oxidixed LDL (*11*) and arachidonate-containing PC (*12*), MDA−lipid adducts also promote the "PS-dependent" prothrombinase assay. These results raise the issue of whether annexin V can be used as an exclusive probe for the detection of cell surface PS and raise the possibility that aldehyde-modified lipids might participate in various "PS-dependent" processes.

MATERIALS AND METHODS

Materials and Routine Procedures. FITC- and biotin-labeled annexin V were from Trevigen (Gaithersburg, MD). Dioleoylphosphatidylcholine (PC), dioleoylphosphatidylethanolamine (PE), and bovine brain PS were from Avanti Polar Lipids, Inc. (Alabaster, AL). *tert*-Butylhydroperoxide (*t*-BHP), ovalbumin (OVA), and rabbit serum albumin (RSA) were from Sigma (St. Louis, MO). Silicon slides were obtained from Aurel GmbH (Landsberg, Germany). Coagulation factors Xa and Va and prothrombin were purified from bovine blood as previously described (*13*). Thrombin-specific substrate S2238 was from Chromogenix-Nodia. Lipid concentrations were determined by phosphate assay and tested for purity by thin-layer chromatography in $CHCl_3$/MeOH/H_2O (65/25/4). Red blood cells (RBC) were obtained from healthy volunteers by venipuncture into acid/citrate/dextrose. The blood was washed in saline, the buffy coat was removed, and the RBC were resuspended in Tris or phosphate-buffered saline (TBS or PBS) to 10% (v/v). MDA was generated from its bis(dimethyl)acetal (Aldrich, St. Louis) by acid hydrolysis in 10 volumes of 1 N HCl at 0 °C for 2 h. The solution was then brought to pH 7.0 with 10 N NaOH and the concentration of the aldehyde estimated from the absorption at 267 nm (ϵ = 34 000) (*14*). MDA antibodies were produced in

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[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; *t*-BHP, *tert*-butylhydroperoxide; MDA, malondialdehyde; MDA−PE, malondialdehyde−phosphatidylethanolamine; MDA−PS, malondialdehyde−phosphatidylserine; RBC, red blood cells; RSA, rabbit serum albumin.

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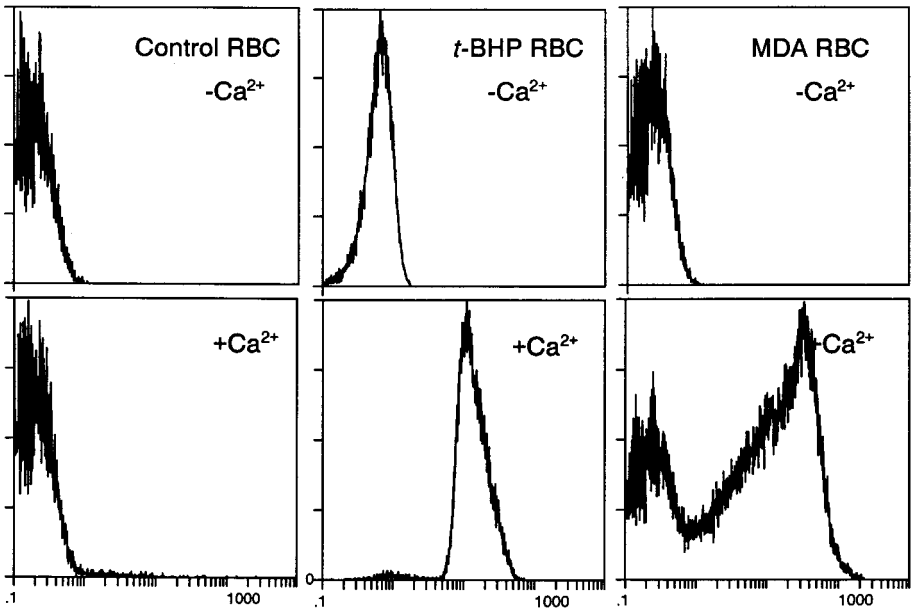

FIGURE 1: Fluorescent-activated cell sorter (FACS) analysis of annexin V binding to oxidized RBC. Control, *t*-BHP-treated (2 mM for 2 h at 37 °C), and MDA-treated (5 mM for 2 h at 37 °C). RBC were incubated with FITC-labeled annexin V in the presence (lower panels) or absence (upper panels) of Ca^{2+} (1 mM).

Table 1: Mass Analysis of MDA−PE Derivatives Obtained from MDA-Treated RBC[a]

			derivative I		derivative II		derivative III	
PE	MW	[M−H]	M−H+[132]	$+Na^{+1}$	[M−H+53]	$+Na^{+1}$	[M−H+107]	$+Na^{+1}$
12:00	579.8	578.8	710.8	733.8	631.8	654.8	685.8	708.8
14:00	635.9	634.9	766.9	789.9	687.9	710.9	741.9	764.9
16:01	687.9	686.9	**818.9**	841.9	739.9	762.9	793.9	816.9
16:00	692.0	691.0	823.0	846.0	**744.0**	**767.0**	798.0	821.0
16:0−18:2	716.0	715.0	847.0	870.0	**768.0**	791.0	822.0	845.0
16:0−18:1	718.0	717.0	849.0	872.0	**770.0**	793.0	824.0	847.0
16:0−18:0	720.0	719.0	851.0	874.0	772.0	795.0	826.0	849.0
18:03	736.0	735.0	867.0	890.0	788.0	811.0	842.0	**865.0**
18:02	740.0	739.0	8871.0	894.0	**792.0**	815.0	846.0	869.0
16:0−20:4	740.0	739.0	871.0	894.0	**792.0**	815.0	846.0	869.0
18:01	744.0	743.0	875.0	898.0	**796.0**	819.0	**850.0**	873.0
18:0−18:2	744.0	743.0	875.0	898.0	**796.0**	819.0	**850.0**	873.0
18:0−18:1	746.1	745.1	877.1	**900.1**	**798.1**	821.1	852.1	875.1
18:00	748.1	747.1	879.1	**902.1**	800.1	823.1	854.1	877.1
16:0−22:6	764.0	763.0	895.0	918.0	816.0	**839.0**	870.0	893.0
18:0−20:4	768.1	767.1	**899.1**	922.1	820.1	843.1	874.1	897.1
20:04	788.1	787.1	919.1	942.1	840.1	**863.1**	894.1	917.1
18:0−22:4	792.1	791.1	923.1	946.1	844.1	867.1	898.1	921.1
22:06	836.1	835.1	967.1	990.1	**888.1**	**911.1**	942.1	965.1

[a] RBC were incubated with MDA as described under Materials and Methods. The lipids were partially purified by TLC. The fluorescent bands comigrating with standard MDA−PE were extracted and analyzed by mass spectroscopy. The table shows the predicted masses for the three potential PE derivatives of the indicated combinations of acyl chains. Boldface values represent found molecular masses.

mice by repeated immunization with MDA−RSA (50 μg in Freunds' adjuvant). Analysis of the antibodies by solid-phase ELISA showed high titers against both MDA−lipid and MDA−protein adducts. Binding of FITC annexin V to RBC was analyzed by flow cytometry with a Coulter Epics Profile flow cytometer employing EPICS Elite software. Negative ionization mass spectra were recorded by Avanti Polar Lipids on a HP5989A mass spectrometer.

Production of Endogenous Aldehydes by Lipid Peroxidation. Aldehydes generated by lipid peroxidation were estimated as thiobarbituric acid-reactive compounds (*14*). Briefly, RBC (1 mL at 10% packed cells; $\sim 10^9$ RBC) were precipitated on ice for 30 min with trichloroacetic acid (0.25 mL of 1.8 M). The precipitate was removed by centrifugation, and the supernatant was incubated with 0.25 mL of thiobarbituric acid (0.07 M in 0.05 M NaOH) for 15 min in a boiling water bath. After cooling to 20 °C, 0.5 mL of 1 M NaOH was added. The concentration of thiobarbituric acid-reactive compounds was estimated by absorption at 532 nm ($\epsilon = 153\,000$).

Synthesis of MDA Adducts. MDA−protein adducts were synthesized by incubating RSA (10 mg/mL) with MDA (10 mM at 37 °C for 3 h). MDA−PE and MDA−PS standards were synthesized by reacting 38 μmol of freshly prepared MDA with 16 μmol of PE or PS in 3.8 mL of $CHCl_3$/MeOH/H_2O (1/2/0.8) for 3 h at 37 °C. After adding 1 mL of $CHCl_3$ and 1 mL of water, the lower phase was collected, evaporated, and solubilized in $CHCl_3$/MeOH (1/1).

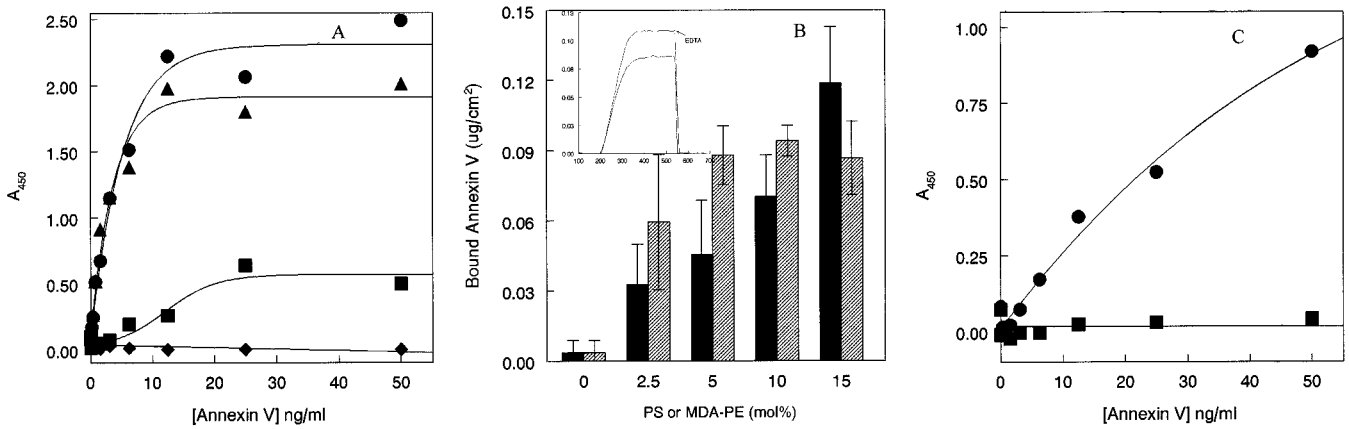

FIGURE 2: Binding of annexin V to MDA adducts. (A) Lipid ELISA. The indicated lipids (25% in PC; 7.6 nmol of lipid/well) were coated on ELISA plates, and annexin binding was determined with biotinylated annexin V: (●) MDA−PE; (■) PE; (◆) PC; (▲) PS. (B) Ellipsometry. Silicon slides were coated with PS or MDA−PE in 85% PC/15% PE. The indicated concentrations of target lipid were substituted for PE. PS, black bars; MDA−PE, gray bars. Inset: Binding time course using 15% PS (upper curve) or MDA−PE (lower curve). (C) Protein ELISA: (●) MDA−RSA and (■) RSA (6 μg/well) were coated on ELISA plates, and annexin binding was determined as described for (A). Ellipsometry studies using MDA-modified proteins could not be done because of insufficient binding to the silicon slides.

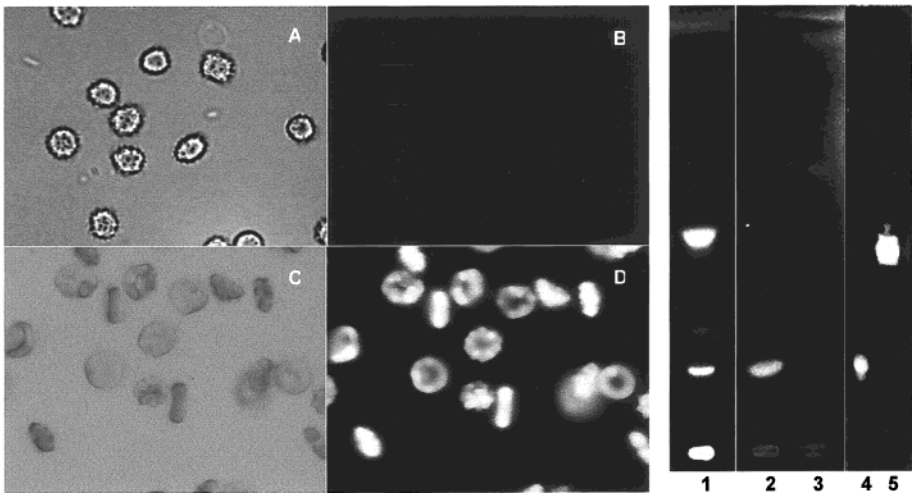

FIGURE 3: Fluorescence microscopy and lipid analysis of MDA-treated RBC. Photomicrographs: Control (A, B) and MDA-treated RBC (C, D) were photographed under phase illumination (left) and fluorescence (right) using 365 and 465 nm excitation and emission bandpass filters, respectively. TLC: lane 1, MDA-treated RBC; lane 2, *t*-BHP-treated RBC; lane 3, control RBC; lane 4, standard MDA−PS; lane 5, standard MDA−PE. Silica gel 60 plates were developed in $CHCl_3$/MEOH/water (65/25/4). The plate was illuminated at ~350 nm and photographed.

RBC Treatment. Increasing concentrations of MDA or *t*-BHP in PBS were added to a 10% (v/v) suspension of RBC and incubated at 37 °C for the indicated times. The cells were then washed with PBS and analyzed as indicated.

Lipid Vesicles. For the prothrombinase assay, dried mixtures of 20 mol % DOPS or DOPE in DOPC were hydrated and sonicated for 10 min at a concentration of 1 mM. MDA was then added to the indicated concentration and incubated at 37 °C. Samples were taken at various time intervals and diluted 1000-fold into the prothrombinase cocktail (carryover amounts of unreacted MDA had no effect on the results).

ELISA Assay. The binding of annexin V to the MDA adducts was determined with biotinylated annexin V. Briefly, 96-well polystyrene ELISA plates were coated with the indicated derivatives overnight at 20 °C. The plates were then blocked with ovalbumin (1%) and incubated with serial dilutions of biotin−annexin V in 1% ovalbumin containing $CaCl_2$ (1 mM) for 2 h at 20 °C. The plates were then washed with TBS/Ca^{2+} (20 mM Tris/150 mM NaCl/1 mM $CaCl_2$, pH 7.4) and incubated with avidin−peroxidase at 0.2 μg/mL (Sigma) for 1 h at 20 °C. The plates were washed and developed with TMB-ELISA (3,3′,5,5′-tetramethylbenzidine base, GIBCO BRL).

Ellipsometric Measurement of Protein Adsorption to Lipid Bilayers. Protein adsorption to planar bilayers was measured by ellipsometry as previously described (*15, 16*). Measurements were performed at a 68° angle of incidence (HeNe laser) (*17*). Planar bilayers were deposited on silicon slides by immersion for 5 min in a stirred suspension of sonicated vesicles (30 μM) in Tris buffer (50 mM Tris, 120 mM NaCl, 3 mM $CaCl_2$, pH 7.5) (*18*). Experiments were performed at 20−22 °C with continuous stirring in a trapezoidal cuvette in Tris buffer (10 mM Tris, 150 mM NaCl, 0.5 mg/mL bovine serum albumin, pH 7.5) containing 3 mM $CaCl_2$.

Prothrombinase Assay. Lipid vesicles were diluted in Tris buffer (10 mM Tris, 150 mM NaCl, 0.5 mg/mL bovine serum

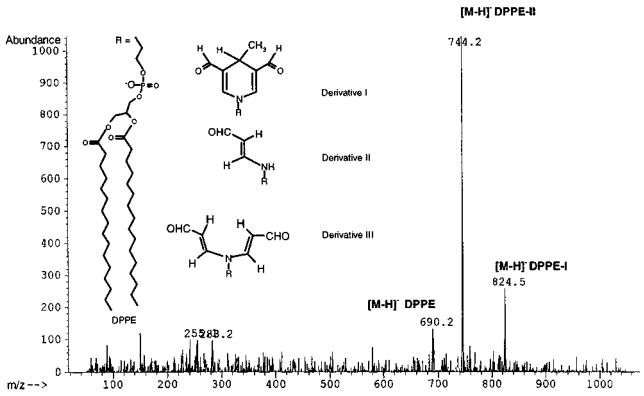

FIGURE 4: Molecular mass analysis of MDA−lipid products. Standard MDA derivatives of DPPE were synthesized as described under Materials and Methods. Negative ionization mass spectra were recorded on a HP5989A mass spectrometer. Mass analysis *m*/*z* calculated for derivative I, 824.97, found 824.5; calculated for derivative II, 744.97, found 744.2. Predicted derivative III was not detected.

albumin, pH 7.5) containing 3 mM $CaCl_2$ (final volume 175 μL). Then 25 μL of factors Xa (2 nM) and Va (20 nM) in the same buffer was added and incubated for 1 min at 37 °C. Subsequently, 50 μL of prothrombin (5 μM) was added to initiate the reaction. After 2 min, 25 μL of the reaction mix was transferred to a cuvette containing 1 mL of TBS/EDTA (50 mM Tris, 120 mM NaCl, 2 mM EDTA, pH 8) and S2238 (0.2 mM). The amount of thrombin generated was determined by the rate of S2238 cleavage to its chromogenic product at 405 nm using a calibration curve obtained with active-site-titrated thrombin.

RESULTS AND DISCUSSION

Lipid peroxidation was initiated in human RBC by incubating the cells with *t*-BHP. FACS analysis of these cells revealed a population of RBC that bound annexin V only in the presence of Ca^{2+} (Figure 1). Since these cells also produced MDA (~1 nmol/10^9 RBC), we tested whether the propensity of the cells to bind annexin V might be due to the formation of MDA−lipid adducts. Figure 1 also shows that direct labeling of RBC with MDA produced a population of cells that bound annexin V, suggesting that the in situ generation of MDA adducts was involved in the binding of annexin to the *t*-BHP-treated RBC.

Several independent assays were carried out to verify that annexin V indeed binds to aldehyde−lipid adducts. Since PE is the most abundant aminophospholipid in cells, MDA−PE was synthesized and tested for its ability to bind annexin V in a solid-phase ELISA assay. The data shown in Figure 2A indicate that MDA−PE bound annexin V in a manner that was indistinguishable from PS. Independent binding studies by ellipsometry also showed that annexin V bound both PS and MDA−PE to similar extents (Figure 2B). Interestingly, annexin V also binds MDA-modified protein (Figure 2C), suggesting that both aldehyde-modified ligands can serve as targets for annexin V.

Examination of MDA-treated RBC by fluorescence microscopy (Figure 3) showed blue fluorescence when excited at ~365 nm. Analysis of lipids extracted from these cells by TLC showed two distinct products that comigrated with standard MDA−PE and MDA−PS (Figures 3 and 4 and Table 1). Surprisingly, *t*-BHP-treated cells did not show blue fluorescence (not shown), and lipid analysis revealed a single fluorescent spot that comigrated with MDA−PS (Figure 3). The apparent absence of MDA−PE could be due to the generation of nonfluorescent aldehyde adducts or the formation of PE/aldehyde/protein cross-links that would preclude extraction of the lipids into organic solvents. This might be due to the relatively low amounts of aldehydes generated in situ (~1 nmol/10^9 RBC) as compared to the concentrations of exogenously supplied MDA (>1 μmol/10^9 RBC). Indeed, analysis of aldehyde antibody binding to *t*-BHP- and MDA-treated RBC showed ~2-fold less binding to the *t*-BHP-treated cells (Figure 5), indicating, nonetheless, that aldehyde adducts are present on the surface of these cells. Because the aldehyde antibodies are not lipid-specific, the observed binding could reflect the presence of MDA−protein adducts. Indeed, annexin V can also bind to aldehyde-modified proteins (Figure 2B).

Because modification of primary amines by aldehydes introduces alterations in the net charge of the target moiety, it was important to differentiate whether the aldehyde antibodies and annexin V share a common target epitope. Solid phase binding assays revealed that antibody binding to lipid was largely dependent on the aldehyde motif because binding was significantly attenuated after $NaBH_4$ reduction (Figure 6A). Unlike antibody, annexin V binding was insensitive to

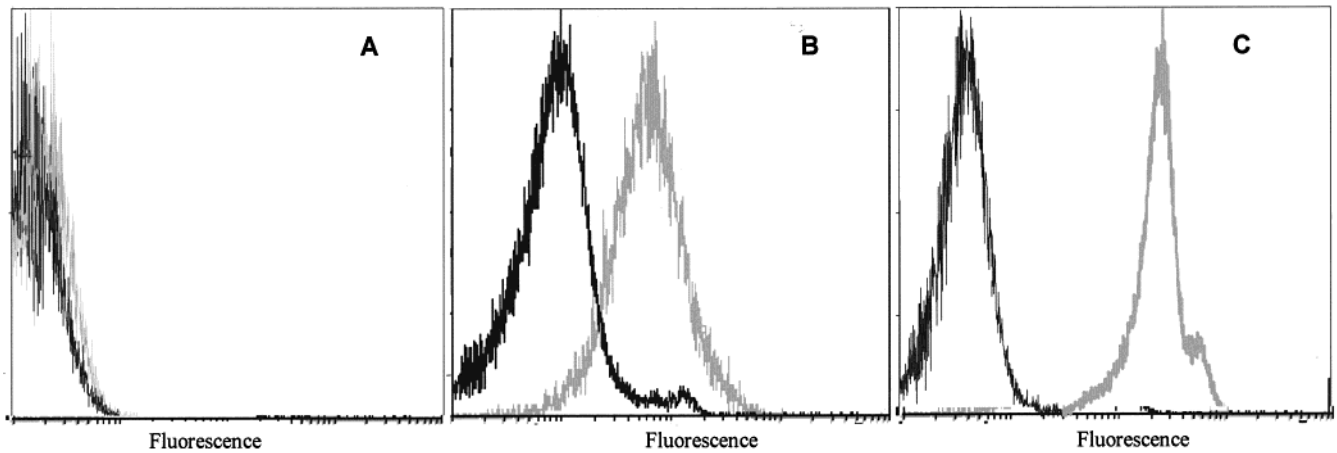

FIGURE 5: Appearance of MDA adducts on the surface of *t*-BHP-treated RBC. Control RBC (A), *t*-BHP-treated RBC (B), and MDA-treated RBC (C) were incubated with normal mouse serum (black trace) or anti-MDA (gray trace). Antibody binding was detected by FACS analysis after staining with FITC-anti-mouse IgG.

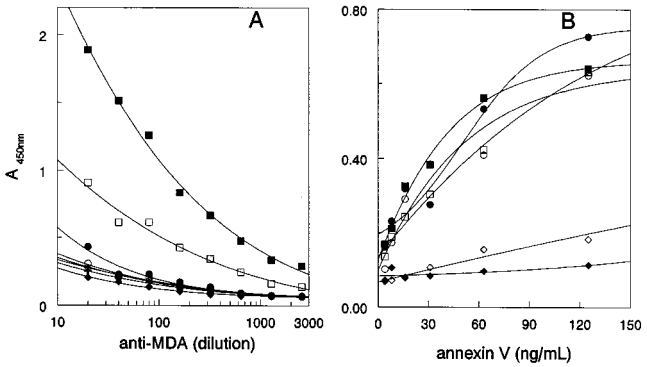

FIGURE 6: Effect of $NaBH_4$ reduction on the binding of MDA antibodies and annexin V to MDA−PE. Antibody binding (panel A): The indicated lipids were deposited on polystyrene plates (6 μg of total lipid well at 25 mol % in PC) overnight. Appropriate wells were treated with 10 mM $NaBH_4$ for 30 min at 20 °C (open symbols). After blocking with OVA (1%), the plates were incubated with serially diluted anti-MDA. Binding was detected using HRP-conjugated anti-mouse IgG/TMB-ELISA. Annexin V binding (panel B). Plates were prepared as described above. After blocking, the plates were incubated with serial dilutions of biotinylated annexin V in 1 mM Ca^{2+} for 2 h at 20 °C. Binding was detected with HRP-conjugated avidin. (●, ○) PS; (■, □) MDA−PE; (◆, ◇) PC.

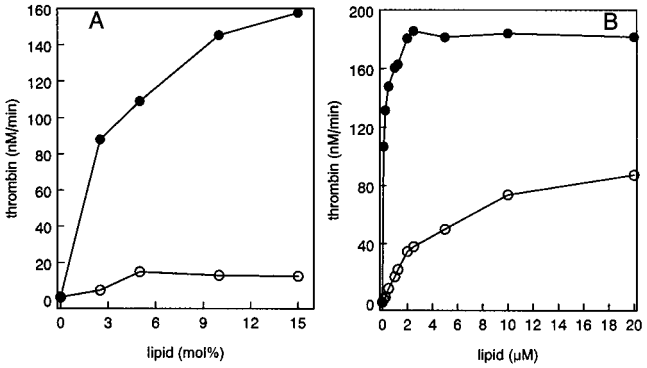

FIGURE 7: Prothrombinase activity of MDA−PE and PS vesicles. (A) Increasing concentrations of PS (●) or MDA−PE (○) (substituted for DOPE) in 2 μM DOPC/DOPE (85/15). (B) Increasing concentrations of vesicles containing 15 mol % MDA−PE (lower curve) or PS (upper curve) in DOPC/DOPE.

reduction (Figure 6B), indicating that binding was dependent on the charge of the target moiety.

Because these results suggest that MDA−PE can substitute for PS in annexin V binding assays, it was of interest to determine whether it could also mimic PS in other "PS-dependent" systems. Figure 7 compares the procoagulant activity of vesicles composed of increasing concentrations of PS and MDA−PE. It can be seen that relatively low concentrations of PS were sufficient to promote thrombin formation. For example, vesicles containing 15 mol % PS were 10-fold more procoagulant than MDA−PE. While MDA−PE lacked significant procoagulant activity at low lipid concentrations, increasing the lipid concentration 10-fold increased thrombin formation by 3-fold (Figure 7B). This "PS-like" activity has also been reported for oxidized LDL (*11*) and arachidonyl-PC (*12*).

In summary, these results indicate that annexin V binds to both PS and aldehyde-modified lipids. Since lipid peroxidation dependent pathways are beginning to be recognized as important initiators of many biological processes including apoptosis (*8*, *19*, *20*), the dogma of PS exposure as determined by annexin V binding should be critically evaluated. Clearly, the development of highly sensitive methods that can differentiate between these moieties is crucial toward understanding the role of aldehyde adducts and lipid asymmetry in apoptosis.

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