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Peroxidation-induced perturbations of erythrocyte lipid organization

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Peroxidation of erythrocyte membrane lipids by hydrogen peroxide perturbs the lipid bilayer and increases phagocytosis by macrophages. This study addresses the underlying mechanism of these processes, and in particular the role of malondialdehyde, a major byproduct of lipid peroxidation. When erythrocytes were treated with hydrogen peroxide or ascorbate/iron to generate malondialdehyde, or with malondialdehyde itself, only those cells treated with hydrogen peroxide showed increased phospholipid spacing and enhanced phagocytosis. This result indicates that the alterations observed are unique to hydrogen peroxide treatment, and that malondialdehyde does not play a role in inducing these changes in surface properties. Comparison of adherence to human umbilical vein endothelial cells and phagocytosis showed that increased phagocytosis was not mirrored by enhanced adherence. This result suggests that two different signals may mediate recognition of erythrocytes by macrophages and by endothelial cells.

Introduction

The lipids of the outer leaflet of the erythrocyte membrane are more tightly-packed [1] and less fluid [2–4] than those of the inner leaflet. When the normal asymmetric distribution of phospholipids across the erythrocyte membrane [5] is disrupted, the outer leaflet becomes less tightly packed [1], more fluid [2] and more hydrophobic [6]. These changes in the biophysical properties of the erythrocyte surface are accompanied by enhanced phagocytosis of lipid-symmetric erythrocytes by macrophages [6]. Similarly, more dense, older erythrocytes have a less tightly packed outer leaflet [7] and are more readily phagocytosed [8] than less dense, younger cells, suggesting that macrophages are able to recognize the altered packing of aged erythrocytes and remove them from the circulation [9]. Finally, erythrocytes treated with H_2O_2 also display less tightly packed exterior leaflets [7], enhanced phagocytosis by macrophages [7,10], and increased adherence to monocytes [11,12], suggesting that accumulation of oxidative damage in aged erythrocytes might affect lipid packing and thereby induce cell clearance from the circulation.

One possible intermediate in the mechanism responsible for peroxidation-induced alteration of lipid packing is malondialdehyde (MDA), a major product of phospholipid peroxidation. This highly reactive, bifunctional molecule can crosslink erythrocyte phospholipids [13] and proteins [14,15] and hence is a likely mediator of peroxidative injury. We test here whether MDA is able to induce alterations in lipid packing and enhance phagocytosis of erythrocytes.

Materials

Erythrocyte treatments

Washed human erythrocytes were suspended at 10^8 cells/ml in phosphate-buffered saline (PBS; 122 mM NaCl, 3 mM KCl, 15 mM Na_2HPO_4, 5 mM KH_2PO_4, 0.1% (w/v) glucose, pH 7.35). To inhibit endogenous catalase activity, 1 mM NaN_3 was added and the suspension incubated for 1 h at 37°C [16] in a shaking

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Abbreviations: MC540, merocyanine 540; MDA, malondialdehyde, PC, phosphatidylcholine; PE, phosphatidylethanolamine; TBA, thiobarbituric acid.

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water bath. H_2O_2 (Sigma) was then added at various concentrations and incubation continued for an additional hour. An aliquot was removed for analysis of MDA, and the remaining cells were washed three times at 4° C with five volumes of PBS containing 0.15% BSA prior to other analyses.

MDA was prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane (Sigma) [16] and was added within 10 min at various concentrations to washed erythrocytes suspended at 10^8 cells/ml in PBS. Since 4 moles of methanol are generated for every mole of MDA, methanol was added to control cells at the appropriate concentrations. All samples were incubated for 2 h at 37° C in a shaking waterbath. An aliquot was removed for analysis of MDA, and the remaining cells were washed three times as above prior to other analyses.

Freshly prepared solutions of L-ascorbic acid (1 M) and $FeSO_4$ (5 mM) were added at 100 : 1 molar ratios at varying final concentrations to washed erythrocytes suspended at 10^8 cells/ml in PBS without glucose. Controls contained either ascorbate alone at 100 mM or $FeSO_4$ alone at 1 mM. All samples were incubated for 3 h at 37° C in a shaking waterbath. An aliquot was removed for analysis of MDA, and the remaining cells were washed three times as above prior to other analyses. The thiobarbituric acid (TBA) assay of Stocks and Dormandy [17] was used to measure the generation of MDA.

Erythrocytes with lipid-symmetric membranes were prepared by lysis and sealing in the presence or absence of Ca^{2+} as previously described [18]. Loss or maintenance of asymmetry was verified by staining with merocyanine 540 [19].

Merocyanine 540 staining

To assess lipid packing, cells were stained with MC540 and fluorescence monitored by flow cytometry as before [20]. Briefly, 10 μl of MC540 (100 μg/ml) were added to $1 \cdot 10^6$ cells in 100 μl of PBS containing 0.15% BSA. After 3 min at room temperature the suspension was diluted by addition of 900 μl of PBS, and fluorescence quantified using a Coulter EPICS V flow cytometer with an Argon laser tuned to 514 nm and equipped with a 515 nm barrier filter and 560 nm dichroic mirror.

Phagocytosis assays

The murine, macrophage-like cell line J774A.1 (American Type Culture Collection) was grown in RPMI (GIBCO) supplemented with 10% fetal bovine serum (FBS; Hyclone). $1 \cdot 10^5$ cells suspended in 0.15 ml of growth medium were plated onto 12 mm diameter glass coverslips and incubated overnight at 37° C. Cultures were washed twice with (serum-free) RPMI and $5 \cdot 10^6$ erythrocytes suspended in 0.1 ml of RPMI added to each coverslip. After 1 h at 37° C, nonadherent erythrocytes were removed by washing three times with 2 ml of RPMI. Adherent, but unphagocytosed erythrocytes were lysed by a 2 min exposure to 0.17 M NH_4Cl at room temperature followed by three washes with RPMI.

Cultures were fixed with methanol, stained for hemoglobin with 3,3′-dimethoxybenzidine in methanol, counterstained with Giemsa stain and counted at 400 × magnification. For each treatment, the number of erythrocytes phagocytosed by 1000 macrophages over triplicate cultures was determined.

Adherence assays

Human umbilical vein endothelial cells were isolated as described by Jaffe [21] and grown in Medium 199 with Earl's salts (GIBCO) containing 25 mM Hepes (pH 7.3), 2 mM L-glutamine, 20% FBS, 125 μg/ml of endothelial mitogen (Biomedical Technologies Inc.), 146 U/ml of heparin-sodium salt, 50 U/ml of penicillin and streptomycin, and 2.5 μg/ml of fungizone (GIBCO). Cells reached confluency within 24 to 48 h and were passaged twice at a 1 : 3 dilution before use. Round glass coverslips (13 mm, Bellco Glass) were coated with 0.2% gelatin, placed in 35 mm petri dishes, overlaid with 100 μl of cells suspended in endothelial cell growth medium at a concentration of 10^6 cells/ml, and incubated at 37° C for 2 h, resulting in confluent monolayers. Two ml of endothelial cell growth medium lacking endothelial mitogen and heparin was added to each dish and cultures used within 1 to 5 days.

Coverslip cultures were rinsed by dipping in Hanks' buffered saline solution (130 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 0.25 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM $NaHCO_3$, 6 mM glucose) containing 0.5% human serum albumin (Sigma) (HBSS + HSA) at room temperature and glued (Krazy Glue) to an 18 mm round glass coverslip (Bellco Glass), forming a transparent seal. The endothelial cell monolayer (small coverslip only) was overlaid with 100 μl of erythrocytes suspended at a concentration of 10^7 cells/ml in room temperature HBSS + HSA and incubated at 37° C for 30 min, during which time the erythrocytes settled onto the endothelial cells forming a single monolayer. The double coverslip was then inverted over a well of a 24-well culture dish (GIBCO) filled with HBSS + HSA such that the 18 mm coverslip was supported by and formed an airtight seal with the edges of the 17 mm well. The dish was centrifuged at 110 × g at room temperature for 3 min in a Centra 8 centrifuge (IEC) using rotor No. 216. After centrifugation the coverslips were removed from the culture dish, immersed in petri dishes containing HBSS + HSA, observed at 200 × with an Olympus CK inverted microscope, and the number of erythrocytes in 10 random fields at different locations on the coverslip were counted and summed.

Phospholipase digestion and lipid analyses

In general, the procedures outlined in Williamson et al. [1] were followed. Cell lysis following phospholipase A_2 digestion was determined spectrophotometrically at 418 nm. Phospholipids were extracted according to the procedure of Rose and Oklander [22]. The organic phase of each sample was collected and evaporated to dryness under a stream of nitrogen. Samples were redissolved in a fixed minimum volume of chloroform/methanol (2:1, v/v) and an aliquot removed for determination of recovery of phospholipids by phosphate analysis [23]. The remainder of the concentrated lipid extracts was prefiltered through 0.45 μm Nylon 66 membrane, evaporated to dryness, dissolved in spectral grade chloroform/methanol (2:1, v/v) and analysed by HPLC (Scientific Systems, Inc.). Lipids were separated on a 4.6 × 150 mm 5 μm soft-seal silica column (Scientific Systems, Inc.) using a linear gradient from 88 to 20% acetonitrile/water (80:20, v/v) in acetonitrile, formed between 10 and 45 min. Flow rate was 2 ml/min, the eluate passing through an 8 μl flow cell monitored for absorbance at 206 nm. Peaks were identified by comparison with retention times of phospholipid standards analyzed under identical conditions. Peak areas were normalized to sphingomyelin and the percentage of phospholipids digested calculated by comparing these values for digested versus undigested samples.

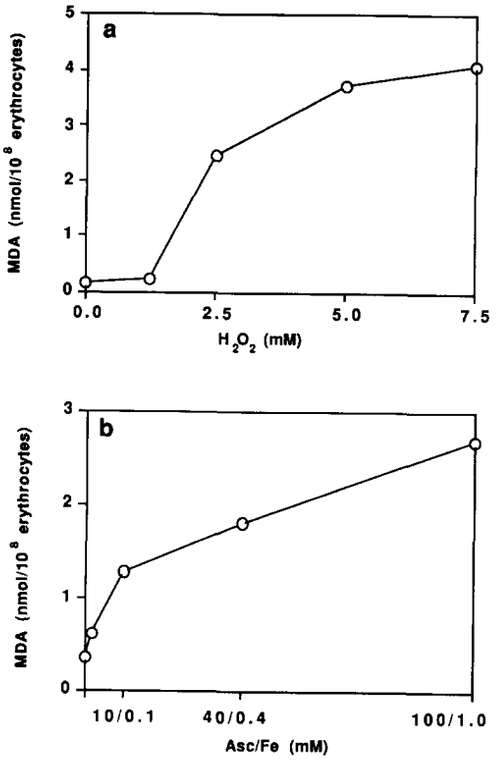

Fig. 1. MDA production by erythrocytes treated with oxidants. Erythrocytes (10^8/ml) were incubated at 37°C with varying concentrations of (a) H_2O_2 and (b) Asc/Fe. At specified times, samples were withdrawn and assayed for MDA content.

Results

Peroxidation of phospholipids generates MDA and several MDA-like aldehydes and ketones, all of which react with TBA [24]. However, for the sake of simplicity, TBA reactivity will be equated with MDA, since MDA is the major reactive product generated. As shown in Fig. 1a, treatment of erythrocytes with increasing concentrations of H_2O_2 generates increasing amounts of MDA to a plateau of 4.1 nmol per 10^8 erythrocytes at 7.5 mM H_2O_2.

The impermeant fluorescent probe MC540, which binds preferentially to loosely packed bilayers [25], was used to confirm that H_2O_2 treatment of erythrocytes alters phospholipid packing in the exterior leaflet of the cell membrane. The flow cytometric profiles of cells stained with MC540 (Fig. 2; left panels) show that control erythrocytes treated with azide but not with H_2O_2 exhibited a single peak of low fluorescence intensity, just as untreated erythrocytes. Treatment with azide and H_2O_2 produced some cells which stained more intensely with MC540. With increasing concentrations of H_2O_2, the number of these cells, and the intensity of their fluorescence, increased. At 7.5 mM nearly all cells exhibited increased fluorescence. Higher concentrations of H_2O_2 did not produce any further alteration in this profile. Treated but unstained cells were not fluorescent at the instrument settings used to detect MC540 fluorescence. These results confirm earlier work [7] suggesting that H_2O_2 treatment of erythrocytes results in a looser packing of the exposed phospholipids in the cell membrane.

To determine whether the MDA levels generated by H_2O_2 treatment can alter lipid packing, erythrocytes were treated with MDA, freshly prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane. As seen in Fig. 2 (right panels), treatment with MDA at a concentration equivalent to that generated by treament with 7.5 mM H_2O_2 (0.02 mM MDA; see Table I) did not alter the packing of phopsholipids as assessed by MC540 staining. Even concentrations of MDA as high as 25 mM, over 100-times higher than the maximal amount generated by H_2O_2 treatment, did not alter the MC540 staining profile (data not shown). To ensure that MDA was not interacting with MC540 and destroying or quenching its fluorescence, MDA was added to cells treated with H_2O_2 just prior to or immediately after staining with MC540; no decrease in the intense fluorescence of the cells was seen.

The possibility remains that MDA must be generated endogenously by lipid peroxidation rather than being simply added exogenously, to affect packing. To test this possibility, ascorbate/iron (Asc/Fe) treatment [26] was used to induce peroxidation of lipids, thereby endogenously generating MDA. As seen in Fig. 1b, treat-

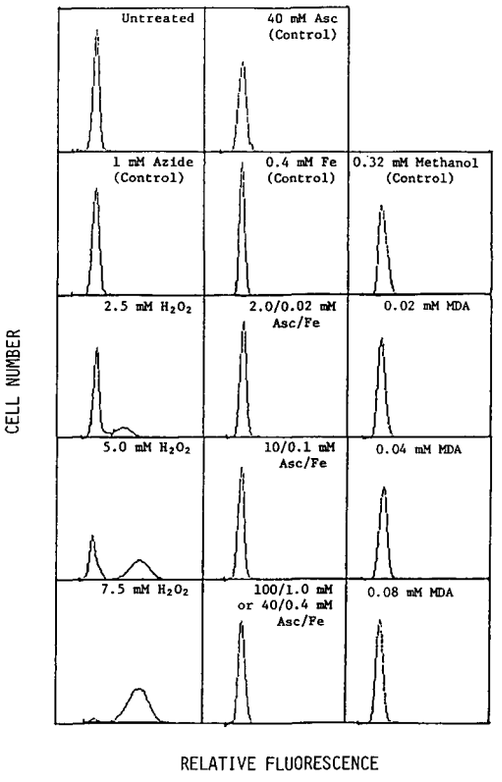

Fig. 2. Effect of oxidants on lipid packing of erythrocytes measured by MC540 fluorescence. Erythrocytes (10^8/ml) were incubated at 37°C with increasing concentrations of H_2O_2 (left panel), Asc/Fe (center panel) and MDA (right panel). Following incubations, treated and untreated cells were withdrawn at specifed times, washed, stained with MC540, and analysed by flow cytometry for their relative fluo - rescence.

ment of erythrocytes with increasing concentrations of Asc/Fe resulted in the generation of increased amounts of MDA up to a level comparable to that generated by 2.5 mM H_2O_2. However, unlike H_2O_2, Asc/Fe treatment did not alter staining by MC540 (Fig. 2, center panels), indicating that the alteration in phospholipid packing is specific to treatment with H_2O_2, and cannot be mimicked by MDA, whether added exogenously or generated endogenously by treatment with Asc/Fe.

These results raise the question of whether the enhanced phagocytosis of H_2O_2-treated erythrocytes is a result of their looser phospholipid packing, or a consequence of other damage induced by MDA. If looser phospholipid packing is responsible, then cells treated with MDA or Asc/Fe, whose packing has not been altered, should not be more susceptible to phagocytosis. To investigate this issue, treated cells were added to monolayers of the murine macrophage-like cell line J774A.1, previously shown to behave like human monocyte-derived macrophages in phagocytosis assays [6,27]. As seen from the representative experiment presented in Table I, phagocytosis increased in a dose-dependent fashion with H_2O_2 treatment. In contrast, macrophages phagocytosed erythrocytes treated with MDA no more efficiently than control cells. Similarly, treatment with Asc/Fe did not result in a comparable increase in phagocytosis. These results indicate that the damaging effects of MDA do not include immediate enhancement of erythrocyte phagocytosis.

Lipid-symmetric erythrocytes in which the packing of exterior phospholipids has been loosened are not

TABLE I

Effect of treating erythrocytes with oxidants on adherence to endothelial cells and on phagocytosis by macrophages

Oxidant	Concn. (mM)	MDA present (nmol/10^8 erythrocytes)	Adherence ratio		Phagocytosis ratio	
			treated/ untreated	treated/ control [b]	treated/ untreated	treated/ control [b]
None						
Untreated erythrocytes		0.2	1.0		1.0	
Lipid-symmetric		0.2	10.4		n.d.	
Azide (control)	1.0	0.2	1.0	1.0	0.6	1.0
H_2O_2	2.5	2.5	0.7	0.7	4.6	7.3
	5.0	3.7	0.8	0.8	6.9	10.9
	7.5	4.1	0.7	0.7	11.8	18.4
Methanol (control)	0.32 [a]	0.2	1.4	1.0	2.6	1.0
MDA	0.02	4.2	0.7	0.5	1.2	0.5
	0.04	9.6	1.6	1.1	1.0	0.4
	0.08	18.4	0.7	0.5	1.0	0.4
Asc (control)	40.0	0.5	1.9	1.0	1.2	1.0
Fe (control)	0.4	0.2	1.3	1.0	1.1	1.0
Asc/Fe	2.0/0.02	0.6	1.3	0.9	0.8	0.8
	10.0/0.1	1.3	1.5	1.1	2.1	1.8
	40.0/0.4	1.8	0.6	0.4	1.8	1.5
	100.0/1.0	2.7	n.d.	n.d.	0.8	1.5

[a] Amount present during treament with 0.08 mM MDA.
[b] Ratios taken to corresponding controls.

TABLE II

Effect of treating erythrocytes with oxidants on recovery and digestion of phospholipids [a]

Oxidant	Concn. (mM)	Hemolysis following digestion (%)	Phospholipid recovery [b]	Phospholipid digested (%)	
				PC	PE
None					
Untreated erythrocytes		0.3±0.01	1.00	71.5±1.1	19.7±2.3
Lipid-symmetric		1.1±0.02	0.87	53.1±3.1	43.2±3.9
Azide (control)	1.0	0.5±0.04	0.88	64.2±3.7	13.7±3.0
H_2O_2	2.5	7.7±2.5	0.67	75.6±5.4	26.0±3.7
	5.0	8.5±3.8	0.59	64.3±0.5	84.6±4.1
	7.5	8.2±3.7	0.44	61.0±2.1	81.6±2.8
Methanol (control)	0.32 [c]	0.2±0.03	0.93	69.5±3.3	24.5±1.1
MDA	0.02	0.4±0.03	0.92	70.6±3.3	21.6±1.2
	0.045	0.2±0.01	0.89	69.6±1.2	17.6±2.0
	0.08	0.3±0.01	0.85	70.0±0.8	21.6±2.6
Asc (control)	40.0	0.6±0.03	0.88	74.0±1.1	26.5±1.5
Fe (control)	0.4	1.2±0.2	0.87	73.0±1.2	23.5±1.5
Asc/Fe	0.2/0.02	1.1±0.2	0.93	71.1±0.8	24.3±2.8
	10.0/0.1	1.4±0.4	0.75	67.3±1.6	15.6±3.2
	40.0/0.4	1.9±0.6	0.59	61.0±1.6	23.3±4.0

[a] Values are an average of five experiments (mean ± S.D.).
[b] Normalized to untreated erythrocytes. Only values for digested samples are presented; those for undigested samples were similar.
[c] Amount present during treatment with 0.08 mM MDA.

only more readily phagocytosed, they are also more adherent to monolayers of endothelial cells [28]. If altered lipid packing is responsible for this increased adherence, then cells treated with H_2O_2 should also adhere more strongly to endothelial cells. To test this possibility, confluent monolayers of human endothelial cells were covered with a single monolayer of treated or untreated erythrocytes. Following incubation at 37°C for 30 min, nonadherent erythrocytes were removed by centrifugation and the adherent cells which remained were counted. As seen from the representative experiment presented in Table I, lipid-symmetric erythrocytes were 10 times more adherent than untreated cells, results similar to those obtained in conventional manual, pipet-washing assays [28]. As might have been expected from the phagocytosis experiments, erythrocytes treated with MDA or Asc/Fe were no more adherent than controls. Surprisingly, however, the adherence of cells treated with H_2O_2 was not significantly increased, and in particular was not comparable to the increase in phagocytosis of the same cells, nor to the increased adherence of symmetric erythrocytes.

Although lipid-symmetric erythrocytes and erythrocytes treated with H_2O_2 share the property of loosely packed exterior leaflets, and are comparably stained with MC540, only the former are more adherent to endothelial cells. This result suggests that a loosely packed lipid leaflet is not sufficient to enhance adherence to endothelial cells, and also suggests that the loose packing of lipids induced by treatment with H_2O_2 does not derive from loss of phospholipid asymmetry. To examine this latter possibility more directly, phospholipase A_2 digestion was used to assess the transbilayer distribution of phospholipids in treated erythrocytes. When untreated erythrocytes were exposed to the lipase, the digested fraction of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Table II), corresponding to the fraction in the outer leaflet, was similar to values reported by others [29,30] and by us [1,18,31,32] for normal erythrocytes. When lipid-symmetric erythrocytes were examined, the amount of PE digested was increased roughly 2-fold as previously reported [18].

As shown in Table II, when erythrocytes were exposed to MDA or Asc/Fe, the fraction of PE digested by phospholipase was marginally elevated, but similar values were observed in the appropriate controls. The fraction of PE digested in H_2O_2 controls was similar to values observed in untreated cells, but in cells treated with H_2O_2, the amount of PE digested was increased in all cases, and especially in cells treated with 5.0 and 7.5 mM H_2O_2. Normally, such a result would be taken as evidence that more PE had become exposed on the outer leaflet of the membrane. However, recovery of phospholipids from these samples (both digested and undigested) was reduced (Table II) and could not be improved by increasing the vigor of mechanical disruption of membranes at each of the isolation and extraction steps. Although recovery of all types of phospholipids was reduced, the reduction (in undigested sam-

ples) was greatest for PE. Since reliable calculation of the fraction of PE digested depends on efficient recovery (see Discussion), no real conclusions can be drawn concerning the transbilayer distribution of PE in these samples.

Discussion

The primary goal of these studies was to clarify the mechanism by which H_2O_2 treatment induces recognition and phagocytosis of erythrocytes by macrophages, and in particular, whether MDA generated by peroxidation of lipids has a role in this process. Our results indicate that MDA, either added externally or generated internally by Asc/Fe treatment, does not induce either an alteration in phospholipid packing or an enhancement of interactions with macrophages. These results are in contrast to those of Hebbel and Miller [10], who reported that treatment with 1 mM MDA resulted in increased phagocytosis of erythrocytes. However, in that study, erythrocytes were added to suspensions of macrophages and incubated for 15 h, while in our assays, erythrocytes were added to adherent macrophages and incubated for only an hour. Since H_2O_2 treatment generated observable differences in erythrocyte phagocytosis in the short term incubations employed here, our results imply that MDA has no role in this process, whatever its effects in the longer term incubations employed by Hebbel and Miller.

As increased spacing of phospholipids accompanies loss of phospholipid asymmetry, it was of considerable interest to determine whether the change in packing induced by H_2O_2 treatment resulted from loss of lipid asymmetry. Unfortunately, no conclusions can be drawn in this regard. Phospholipase digestion of PE was greatly increased in cells treated with H_2O_2. However, if PE were symmetrically distributed in the oxidant treated cells in this study, only 50% should become available for digestion, rather than the 80–85% actually observed. Such high values can result from cell lysis, which allows access of phospholipase A_2 to PE in the inner leaflet, and hemolysis was in fact greater in H_2O_2-treated cells during phospholipase digestion (Table II). However, the extent of hemolysis observed is insufficient to account for the increases in PE digestion realized, and would also have resulted in similar levels of PC digestion, a result which was not observed. H_2O_2-induced hemolysis, therefore, cannot account for the PE digestion levels which were observed.

The higher apparent levels of PE digestion might be a consequence of poor recovery of lipid from the H_2O_2 treated samples, since reliable determination of the fraction of total lipid hydrolyzed necessarily requires that total lipid be recovered. For example, preferential recovery of lyso PE versus PE following digestion would lead to erroneously high values of PE digested. Since there is no way of determining whether PE is or is not preferentially recovered when recovery of lipid is less than quantitative, it is not possible to determine whether asymmetry has been lost in H_2O_2-treated cells. Yet another complication is introduced by the finding that oxidation itself can convert phospholipids to their lyso derivatives [33,34]. These types of considerations may apply to previous reports of H_2O_2-induced loss of lipid asymmetry [16], since neither hemolysis, phospholipid recovery, nor oxidative generation of lysophospholipids were reported.

Although recovery of phospholipid from cells treated with Asc/Fe was also reduced at the highest concentrations used, the results show no indication that either Asc/Fe or direct addition of MDA changed the pattern of lipid hydrolysis by phospholipase. These data imply that the effects of H_2O_2 on PE accessibility, whatever their mechanism, are not mediated by the generation of MDA. Indeed, the combined results presented here tend to discount the importance of MDA in producing the alterations in membrane lipid organization and cell adherence properties seen following treatment with H_2O_2. Rather, they suggest that these effects are specific to treatment with H_2O_2. Whether oxidized lipids themselves, or other oxidation products [35,36] are responsible for inducing these effects will require further experiments for clarification.

An important finding to emerge from these studies is that recognition of erythrocytes by macrophages can be uncoupled from recognition by endothelial cells. In previous studies, lipid-symmetric erythrocytes were found to be both more readily phagocytosed by macrophages [6] and more adherent to endothelial cells [28], suggesting that phagocytosis and adherence by cells of the reticuloendothelial system might occur by a similar mechanism. However, as shown here, cells treated with H_2O_2 were more readily phagocytosed by macrophages but were not significantly more adherent to endothelial cells, suggesting that macrophage recognition occurs by a different mechanism than endothelial cell recognition. These results differ from those of Wali et al. [37] who found that H_2O_2 treatment of erythrocytes increased their adherence to endothelial cells. Whether differences in assay conditions (e.g., the endothelial cells used here were passaged only twice before use, and human serum albumin was present throughout our assays) are sufficient to account for the discrepancy is not clear and remains under investigation.

Since the spacing of exterior phospholipids is increased in both lipid-symmetric and H_2O_2-treated erythrocytes, this alteration may be the common property which induces phagocytosis of these cells by macrophages. However, increased spacing of phospholipids is not sufficient for recognition by endothelial cells, suggesting that some feature of lipid-symmetric cells, but not H_2O_2-treated cells, is required for recognition by

endothelial cells. One such signal might be the exposure of PS on the external surface [38–40] which accompanies loss of lipid asymmetry. It should be noted, however, that a role for PS as an endothelial cell recognition signal does not eliminate PS as a potential stimulator of recognition by macrophages. In fact, evidence that both of these properties may serve as signals for clearance from the circulation has been obtained in vivo using liposome models of the erythrocyte surface [41].

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