Oxidative Damage Does Not Alter Membrane Phospholipid Asymmetry in Human Erythrocytes†

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ABSTRACT: Oxidant-induced damage has been proposed to be the underlying mechanism for loss of membrane phospholipid asymmetry in the erythrocyte membrane. In sickle cell disease, thalassemia, and diabetes as well as in senescent erythrocytes, an apparent correlation between oxidative damage and loss of phosphatidylserine asymmetry has been reported. In the present study, erythrocytes were subjected to various levels of oxidative stress and/or sulfhydryl modifying agents. The transmembrane location of phosphatidylserine (PS) was assessed by FITC-conjugated annexin V labeling and the PS-dependent prothrombinase assay. Transbilayer movement of spin-labeled PS was used to determine aminophospholipid translocase activity. Our data show that cells did not expose PS as the result of oxidative stress induced by phenylhydrazine, hydrogen peroxide, *tert*-butyl hydroperoxide, cumene hydroperoxide, or sulfhydryl modification by *N*-ethylmaleimide (NEM) and diamide, even under conditions that led to severe cellular damage and impairment of aminophospholipid translocase activity. In contrast, the increase of intracellular calcium induced by treatment with calcium and ionophore A23187 leads to a rapid scrambling of the lipid bilayer and the exposure of PS, which can be exacerbated by the inhibition of aminophospholipid translocase activity. Oxidation of the cells with hydrogen peroxide or phenylhydrazine did not affect A23187-induced uptake of calcium, but partly inhibited calcium-induced membrane scrambling. In conclusion, oxidative damage of erythrocytes does not induce exposure of phosphatidylserine on the membrane surface, but can interfere with both aminophospholipid translocase activity and calcium-induced randomization of membrane phospholipids.

The transverse membrane lipid asymmetry is a well-established phenomenon in eukaryotic plasma membranes (Op den Kamp, 1979; Devaux & Zachowski, 1994). Under normal conditions, phosphatidylserine (PS)[1] is completely absent in the outer monolayer of the human red cell membrane. Exposure of PS on the erythrocyte surface leads to an enhancement of coagulative reactions resulting in vaso-occlusive events (Zwaal et al., 1992), and is believed to play a role in recognition and removal of defective cells by macrophages (McEvoy et al., 1986; Schroit & Zwaal, 1991).

Loss of PS asymmetry has been reported to occur in apoptosis (Fadok et al., 1992; Martin et al., 1995), in several hemolytic anemias like sickle cell disease (Tait & Gibson, 1994; Kuypers et al., 1996a), in diabetes (Wilson et al., 1993), and in some cases of β-thalassemia (Borenstain-Ben Yashar et al., 1993; Yuan et al., 1994). Also, it has been connected with sequestration of senescent and stored (in vitro aged) red blood cells (Connor et al., 1994). The apparent correlation of impaired oxidative repair mechanisms and increased oxidant damage (Kuypers et al., 1996b; Jain & Shohet, 1984; Jain, 1985; Shinar & Rachmilewitz, 1990; Wolfe, 1989; Herrmann & Devaux, 1990) with the defects in these erythrocytes has often lead to the suggestion that the oxidative events may be related to the observed loss of phospholipid asymmetry.

Loss of phospholipid asymmetry requires an increased movement of phospholipids from the inner to the outer monolayer to scramble the bilayer organization and expose PS on the outside of the cell. In addition, inhibition of the ATP-driven aminophospholipid translocase, or "flipase", which transports the aminophospholipids from the outer to the inner monolayer of the membrane is needed [for a review, see Devaux and Zachowski (1994)]. An active flipase would potentially be able to compensate for the membrane scrambling and restore the asymmetric distribution. It has been shown that the flipase depends on a reduced sulfhydryl group for its activity, evidenced by its inhibition by *N*-ethylmaleimide (NEM) (Morrot et al., 1989). This apparent sensitivity to sulfhydryl modification, which could be the result of oxidative stress together with a possible decrease of ATP, would predict impaired flipase activity in oxidatively challenged red cells. An increased movement of phospholipids across the bilayer independent of ATP has also been reported as the result of oxidative damage (Haest et al., 1978; Franck et al., 1986). Given the apparent correlation between oxidative events and loss of phospholipid asymmetry, we undertook this study to investigate a direct effect of oxidative

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[1] Abbreviations: NEM, *N*-ethylmaleimide; PS, phosphatidylserine; spPS, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; FITC, fluorescein isothiocyanate; FITC-AV, FITC-labeled annexin V; PBS, phosphate-buffered saline; HBSS, Hanks' buffered saline solution; DI_{max}, maximum deformability index; BSA, bovine serum albumin.

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damage on membrane asymmetry. Erythrocytes were exposed to a panel of sulfhydryl modifying and oxidatively active reagents that cause a spectrum of oxidative damage. Hydrogen peroxide, *tert*-butyl hydroperoxide, and cumene hydroperoxide were used as oxidants that form reactive oxygen species and damage cellular components through free radical mediated events, and invoke damage at different sites of the red cell (Van den Berg et al., 1992). NEM was used as a SH-blocking reagent, known to largely inhibit the flipase activity (Morrot et al., 1989; Devaux & Zachowski, 1994), but apparently not to induce PS exposure (Kuypers et al., 1996a). Diamide was used as a SH-reactive reagent that has been used in particular to cross-link the proteins of the membrane skeleton (Haest et al., 1978), and has been shown to increase transbilayer movement of PC (Franck et al., 1986). Phenylhydrazine was used as an agent that causes lipid peroxidation as well as formation of reactive oxygen species along with formation of Heinz bodies (Jain, 1985). Membrane asymmetry was monitored by measuring the transmembrane location of PS with two independent methods. FITC-labeled annexin V was used as a probe for PS with the strong potency of defining subpopulations of scrambled cells by means of flow cytometry. The prothrombinase assay was used as a sensitive probe to detect small changes in the exposure of PS in the outer monolayer of intact erythrocytes. As a positive control, the calcium ionophore induced increase in cytosolic calcium was used to scramble bilayer lipid organization (Williamson et al., 1992; Kuypers et al., 1996a).

Our results show that oxidative damage to red cells does not lead to loss of PS asymmetry. On the contrary, we provide evidence that certain oxidants may even prevent calcium-induced scrambling in erythrocytes.

MATERIALS AND METHODS

Materials. FITC-labeled annexin V (FITC-AV) was prepared as described before (Kuypers et al., 1996a), or obtained from R&D Systems (Minneapolis, MN) as part of the apoptosis detection kit. Similar results were found with both preparations. Bovine prothrombin, thrombin, factor V, and factor Xa were obtained from Enzyme Research Laboratories Inc., South Bend, IN. Factor V was activated to factor Va by addition of 1 unit of bovine thrombin for each 1 unit of factor V. Chromogenic substrate S2238 was from Chromogenix (Pharmacia Hepar, Franklin, OH). *N*-Ethylmaleimide (NEM) and cumene hydroperoxide were obtained from Aldrich (Milwaukee, WI). Diamide, phenylhydrazine, *tert*-butyl hydroperoxide, and sodium azide were from Sigma (St. Louis, MO). Hydrogen peroxide (30%) was obtained from Fisher Scientific (Pittsburgh, PA) or Sigma. All oxidant reagents were dissolved at 100 mM in PBS on the day of use, except cumene hydroperoxide which was dissolved in ethanol at 500 mM. Hanks' buffered saline solution (HBSS, without calcium chloride, magnesium sulfate, phenol red, and sodium bicarbonate) and calcium ionophore A23187 were obtained from Sigma. Fluo-3-AM was purchased from Molecular Probes (Eugene, OR) and dissolved at a concentration of 1.0 mM in dimethyl sulfoxide containing 20% Pluronic-127 (Molecular Probes).

Erythrocytes. Human venous blood was drawn from healthy donors, after informed consent, in EDTA or heparin as anticoagulant. Cells were pelleted by centrifugation at 1000*g* and washed 3 times with PBS (phosphate-buffered saline: 10 mM Na^+/K^+-phosphate, 145 mM NaCl, pH 7.4). The washed packed cells were adjusted to a hematocrit of 50% in PBS.

Induction of Oxidant Damage. Erythrocytes were incubated at 37 °C for 1 h at 40% hematocrit in PBS in the presence of either 0.5 or 5 mM (final concentration) of each oxidant (as specified in the figures). NEM was also used at a 10 mM final concentration. Hydrogen peroxide induced oxidation was always performed in the presence of 4 mM sodium azide to inhibit catalase activity.

After the incubation, the cells were pelleted at 10000*g* for 10 s, and the supernatant was collected to measure the degree of hemolysis, by comparing the hemoglobin concentration in the supernatant to that in the original cell suspension, by measuring the absorbance at 414 nm. The cells were washed 3 times in a 5-fold volume of PBS, and kept on ice for analysis.

Calcium and Ionophore Treatment of Erythrocytes. Calcium and ionophore treatment has been shown to induce membrane lipid scrambling in normal erythrocytes. Oxidant-pretreated erythrocytes were subjected to this treatment to investigate the level of scrambling in these cells compared to control-incubated cells. The cells were incubated at 16% hematocrit for 1 h at 37 °C in the presence of 1.0 mM $CaCl_2$ and 4 μM calcium ionophore A23187 (final concentration). The incubation was stopped with EDTA, and the ionophore was back-extracted with bovine serum albumin (BSA) exactly as described earlier (Kuypers et al., 1996a).

Fluo-3-AM Loading of Erythrocytes. To monitor the increase in cytosolic calcium as the result of incubation with calcium and ionophore, erythrocytes were loaded with the calcium indicator dye Fluo-3.

Control erythrocytes and oxidatively damaged erythrocytes were resuspended at 0.1% hematocrit in a buffer containing 10 mM Hepes, 70 mM NaCl, 80 mM KCl, 0.15 mM $MgCl_2$, 0.1 mM EGTA, 10 mM inosine, and 5 mM pyruvic acid, pH 7.4. The cells were incubated for 2 h at 37 °C in the presence of 1 μM Fluo-3-AM. Subsequently the cells were washed with buffer and resuspended at 0.2% hematocrit in a buffer containing 10 mM Hepes, 70 mM NaCl, 80 mM KCl, 0.15 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1 mM EGTA, pH 7.4. Calcium ionophore A23187 was added to a final concentration of 0.25 μM. After 60 min of incubation at room temperature, the cells were pelleted, and the supernatant was removed. The cells were resuspended to 10^6 cells per 150 μL in buffer containing 2.5 mM $CaCl_2$ and directly analyzed by flow cytometry as described for FITC-AV.

Ektacytometry. Oxidant stress in erythrocytes generally leads to decreased deformability of the cells, which can be monitored by ektacytometry (Kuypers et al., 1990). The measurements were conducted with a Technicon ektacytometer (Technicon, Tarrytown, NY). The instrument and method for analysis of cellular deformability have been described in detail previously (Kuypers et al., 1990). Data were acquired on an Apple Macintosh computer (Apple, Cupertino, CA) for automated data analysis.

Cellular Thiol. To obtain information on the state of oxidative stress in treated erythrocytes, the total thiol content of the cells was assessed with DTNB in the supernatant of an erythrocyte precipitate in metaphosphoric acid (6%).

ATP. The ATP content of erythrocyte was measured with a luciferin/luciferase bioluminescence assay using an ATP

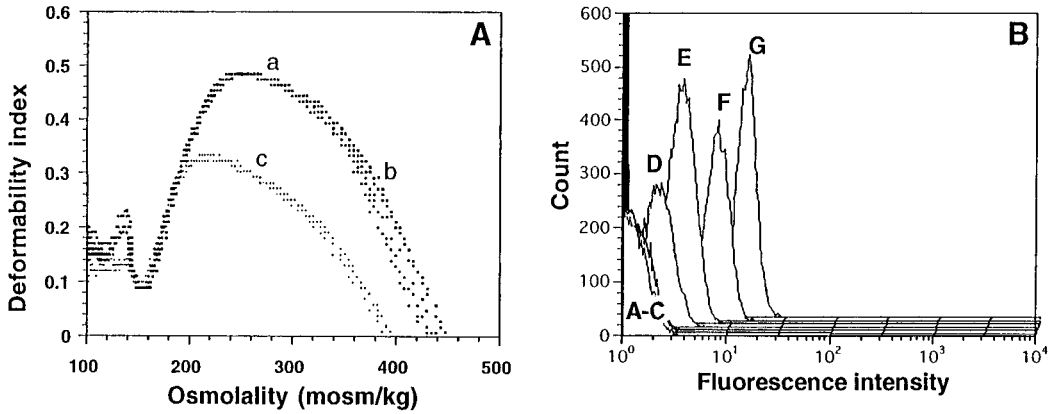

FIGURE 1: Cellular damage as a result of oxidation or sulfhydryl modification. (A) Typical osmotic deformability profile of oxidized erythrocytes. Erythrocytes were incubated at a hematocrit of 40% for 1 h at 37 °C in the absence (curve a) or presence of 4 mM sodium azide and 0.5 mM (curve b) or 5 mM (curve c) hydrogen peroxide as described under Materials and Methods. (B) Flow cytometric determination of autofluorescence of control erythrocytes (A) or cells treated with 5 mM NEM (B), diamide (C), *tert*-butyl hydroperoxide (D), cumene hydroperoxide (E), phenylhydrazine (F), and hydrogen peroxide (G).

bioluminescent assay kit (Sigma, St. Louis, MO), after precipitation of the cells with trichloroacetic acid (6%).

Aminophospholipid Translocase Activity. The spin-labeled PS used contains a short nitroxide-labeled fatty acyl group at the *sn*-2 position. The partial solubility of this probe in water facilitates the incorporation into the red blood cell membrane and enables the measurement of lipid translocation by determination of the fraction of probe in the outer monolayer that can be extracted by BSA (Calvez et al., 1988). To minimize hydrolysis of the spin-labeled PS, erythrocytes were incubated in buffer containing 5 mM diisopropyl fluorophosphate prior to labeling (Calvez et al., 1988). 1-Palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine (spPS) was synthesized as described before (Morrot et al., 1989), dried from a chloroform solution, and resuspended in buffer. Labeling was performed at a 30% hematocrit with 10 μM probe in a buffer containing 10 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM EGTA, 10 mM glucose, 10 mM inosine, 10 mM adenosine, and 10 mM pyruvate, pH 7.4. Incubation in this buffer maintained the ATP level in the cells during the redistribution of spPS from the outer to the inner monolayer (D. Geldwerth, unpublished observations). The amount of probe on the outside of the cell was determined by back-exchange using BSA. Quantitation of the spin-label was performed on a JEOL-1REX EPR spectrometer (JEOL USA, Peabody, MA).

Annexin V Labeling of Erythrocytes and Flow Cytometric Analysis. Erythrocyte labeling and data acquisition were performed essentially as described earlier (Kuypers et al., 1996a). Briefly, erythrocytes were resuspended at 0.04% hematocrit in HBSS containing 1.5 mM $CaCl_2$ and labeled with 60 ng/mL FITC-AV (final concentrations). After an incubation of 30 min at room temperature, the cells were pelleted at 10000*g* for 10 s, and the supernatant was removed. The cells were resuspended to approximately 10^6 cells per 250 μL of buffer in HBSS containing 2.5 mM $CaCl_2$.

Flow cytometry was carried out with a Becton Dickinson FACScan flow cytometer equipped with LYSYS II software, and the data analysis was performed using CellQuest for Macintosh. Forward and sideward scatter profiles were used to define the region of the population of intact red cells. The percentage of annexin V positive erythrocytes was determined from the fluorescence signal in excess of that obtained with a negative (unlabeled) control, which was run for each sample to account for autofluorescence properties of the cells after some of the incubations.

Prothrombinase Assay. The prothrombinase assay, originally designed by Bevers et al. (1982), was used to obtain semiquantitative information on the orientation of PS in cell membranes and was performed essentially as described earlier (De Jong & Ott, 1993). Briefly, intact oxidant-treated and control cells were resuspended at 4% hematocrit in PBS. A 25 μL aliquot of these suspensions was assayed with 0.5 unit/mL factor Va, 0.25 unit/mL factor Xa, and 1.3 units/mL prothrombin (final concentrations) in a total volume of 500 μL in buffer containing 10 mM Tris, 136 mM NaCl, 1.6 mM KCl, 4 mM $CaCl_2$, and 0.5 mg/mL human serum albumin, pH 7.9. At timed intervals of 2 and 4 min after addition of prothrombin, aliquots of 25 μL were transferred to 1 mL of a buffer containing 2 mM EDTA to stop the reaction, and the amount of thrombin formed was assessed spectrophotometrically using the chromogenic substrate S2238.

Ionophore-treated or hemolyzed cells were usually resuspended at an equivalent of 0.1% hematocrit in PBS, but further assayed under identical conditions as intact cells.

After completion of the timed assay, the reaction was stopped by addition of EDTA to a final concentration of 10 mM, and the suspension was centrifuged at 10000*g* for 10 s. The supernatant was sampled to determine hemolysis by comparing the hemoglobin concentration (measured by the absorbance at 405 nm) to an equivalent amount of cell hemolysate.

RESULTS

In the present study, we aimed to induce mild and severe oxidative damage in erythrocytes. The degree of cell deformability as verified by ektacytometry was shown to be significantly reduced in cells treated with oxidant as shown for hydrogen peroxide in Figure 1A. The maximum deformability index (DI_{max}) at 290 mosm was calculated relative to untreated cells for the different oxidative conditions (Table 1). No significant reduction in DI_{max} was observed after treatment of the cells with 0.5 mM oxidant, indicating a mild level of oxidation. In contrast, the higher concentrations of oxidant (5 or 10 mM) led to a significant decrease in DI_{max}, reflecting oxidatively damaged red cells. Analysis of these

Table 1: Oxidant Damage of Erythrocytes Induced by 1 h Treatment with Various Oxidants As Reflected by a Change in the Maximum of Deformability Index Measured between 200 and 300 mosm (DI_{max})[a]

oxidant	concentration (mmol/L)	DI (%)
control	0	100
control incubated 1 h at 37 °C	0	103 ± 2
NEM	0.5	104
	10	53
diamide	0.5	100
	5	<30
phenylhydrazine	0.5	110
	5	<20
hydrogen peroxide	0.5	100
	5	67
tert-butyl hydroperoxide	0.5	109
	5	<40
cumene hydroperoxide	0.5	98
	5	76

[a] The DI_{max} for different conditions is expressed as a percentage relative to the untreated control.

cells by flow cytometry indicated an increase in autofluorescence as the result of oxidative damage (Figure 1B). Modification of sulfhydryl groups did not lead to a change in fluorescence intensity as compared to control red cells. In contrast, treatment with 5 mM *tert*-butyl hydroperoxide, cumene hydroperoxide, phenylhydrazine, and hydrogen peroxide led to increasing shifts in autofluorescence as compared to control cells (Figure 1B). These data indicate the formation of fluorescent products under our experimental conditions.

Determination of the cellular thiol content confirmed differences between oxidative treatments, showing that in most cases the thiol content was only reduced when high concentrations (5 or 10 mM) of oxidant had been used (Figure 2A). This reduction in cellular thiol reflects changes in glutathione as well as the state of thiol-containing proteins and will be in part responsible for the observed changes in the ektacytometric deformability profile as the result of membrane skeleton alterations.

Cellular ATP levels (Figure 2B) were reduced after oxidant treatment to 40−80% of normal ATP levels. This was in part due to the 1 h incubation at 37 °C, which by itself reduced the ATP content to less than 90% of control. Hemolysis after oxidant treatment was usually lower than 0.8%, except after incubation with 5 mM cumene hydroperoxide, which caused progressive hemolysis of the cells.

Phosphatidylserine asymmetry was determined by both annexin labeling and determination of the increase in prothrombinase activity. The prothrombinase assay was found to correlate well with annexin labeling as shown in Figure 3. Fractions of cells with scrambled PS asymmetry, induced by treatment with NEM and subsequently with calcium and ionophore, were mixed with normal cells in various ratios. An increase in the population of annexin positive cells was linearly associated with an increase in prothrombinase activity induced by this cell mixture, indicating a direct relationship between the number of cells that expose PS in a population and the activation of the prothrombinase complex.

Annexin labeling of normal and oxidatively damaged cells (Table 2) revealed that the fraction of annexin positive cells increased by no more than 2-fold after treatment with any oxidant, even at concentrations that caused severe oxidative damage. Average labeling did not exceed 2% of the cells, even when cells were incubated after oxidative damage for several hours. These data indicate that PS did not relocate from the inner to outer monolayer in these cells despite severe oxidative damage.

Similarly to annexin labeling, the prothrombinase activity did not increase significantly in the presence of cells treated with any of the described oxidants (Table 2). Observed slight increases in activity compared to activity in the presence of control incubated cells could always be accounted for by a parallel increase in the level of hemolysis as determined after the assay. Hemolysis exposes the inner bilayer of the cell and provides a PS-rich surface for the prothrombinase complex. As deduced from an assay performed with fractions of cells that had been completely hemolyzed in water, each percent of hemolysis could enhance the prothrombinase activity by 0.56 ± 0.13 unit/μL thrombin per minute in packed cells.

To exclude the possibility that the presence of the oxidant could have modified the FITC or annexin protein moiety and thus impair the method of labeling, cells were treated with 5 mM of either hydrogen peroxide or diamide. Subsequently the cells were resuspended in HBSS containing 1.5 mM $CaCl_2$ and 60 ng/mL AV. After an incubation of 30 min, the cells were pelleted, and the supernatant was collected. This supernatant was used to label erythrocytes that were known to have a scrambled PS asymmetry induced by treatment with NEM and calcium and ionophore as described under Materials and Methods. The cells labeled this way became fluorescent exactly as after labeling with fresh FITC-AV (data not shown), indicating that both the annexin protein and the FITC label were still intact after contact with oxidatively modified cells.

To control whether the prothrombinase assay was capable of measuring PS exposure in these cells, control and treated cells were completely hemolyzed in water to induce exposure of inner monolayer phospholipids. The prothrombinase activity in the presence of these structures increased to 52 ± 16 units/μL thrombin per minute per hemolyzed packed cell volume, independent of the treatment used. This demonstrates that the assay method was not affected by sulfhydryl modification or oxidation, and confirmed the absence of PS in the outer monolayer of the treated cells.

Taken together, only a small fraction of (severely) oxidatively damaged cells expose PS.

An active flipase could be in part responsible for these observations. The flipase could rapidly transport PS from the outer to the inner monolayer, restoring PS asymmetry and leading to a lack of labeling with annexin or activation of the prothrombinase. The ATP levels, although reduced (Figure 2B), might still be sufficient to sustain flipase activity. Similarly, the partly reduced thiol levels do not warrant inhibition of the flipase. Hence, we measured the redistribution of spin-labeled PS (spPS) across the bilayer as a measure of flipase activity under our experimental conditions.

Figure 4A shows the effect of NEM on the transbilayer redistribution of spPS at 37 °C. While spPS moves rapidly from the outer to the inner monolayer in control cells, in NEM-treated cells this movement is grossly impaired. At 10 min, virtually all spPS is located in the inner monolayer of control cells while approximately 10% has transferred to

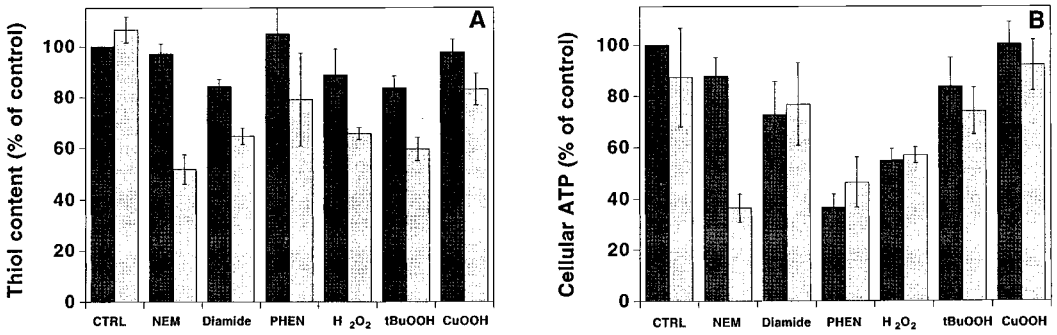

FIGURE 2: Oxidant-induced damage in erythrocytes. Total thiol (A) and ATP (B) were determined as described under Materials and Methods, and expressed relative to untreated control. Both plots show data for control cells (CTRL) that were untreated (dark bars) or incubated for 1 h at 37 °C in PBS (light bars); for cells treated with 0.5 (dark bars) or 10 mM (light bars) NEM; and for cells treated with 0.5 (dark bars) or 5 mM (light bars) diamide, phenylhydrazine (PHEN), hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (tBuOOH), or cumene hydroperoxide (CuOOH) as described under Materials and Methods.

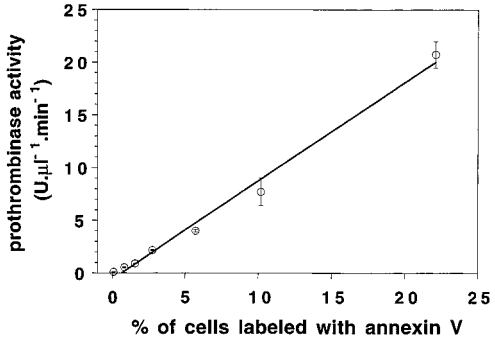

FIGURE 3: Relationship between the percentage of red cells that label with FITC−annexin V and prothrombinase activity in the presence of these cells, expressed as units per microliter of thrombin formed per minute per packed cell volume of erythrocytes (80% hematocrit). Erythrocytes with scrambled PS asymmetry induced by treatment with NEM and calcium and ionophore (see Materials and Methods) were mixed in various ratios with untreated cells. These cell mixtures were subjected to labeling with annexin V and in parallel used in the prothrombinase assay. The correlation of the linear fit was determined to be $R = 0.995$.

Table 2: PS-Dependent FITC−Annexin V Binding to and Prothrombinase Activity in the Presence of Erythrocytes Treated with Oxidants and Sulfhydryl Modifying Agents As Specified (1 h at 37 °C)[a]

oxidant	concentration (mmol/L)	labeled cells (%)	thrombin formation (unit·μL^{-1}·min^{-1})
control	0	0.27 ± 0.24	0.49 ± 0.14
control incubated 1 h at 37 °C	0	0.78 ± 0.74	0.50 ± 0.16
NEM	0.5	0.32 ± 0.43	0.47 ± 0.04
	5	0.83 ± 1.01	nd
	10	0.21 ± 0.17	0.60 ± 0.04
diamide	0.5	0.76 ± 0.29	0.81 ± 0.04
	5	0.71 ± 0.69	0.61 ± 0.02
phenylhydrazine	0.5	0.36 ± 0.11	0.37 ± 0.03
	5	0.17 ± 0.09	0.55 ± 0.03
hydrogen peroxide	0.5	0.28 ± 0.07	0.69 ± 0.07
	5	0.02 ± 0.02	0.45 ± 0.09
tert-butyl hydroperoxide	0.5	1.47 ± 1.30	0.77 ± 0.05
	5	1.34 ± 1.89	0.83 ± 0.03
cumene hydroperoxide	0.5	1.87 ± 1.89	0.62 ± 0.04
	5	0.08 ± 0.08	0.70 ± 0.04
NEM + hydrogen peroxide	10/5	0.42 ± 0.07	nd
NEM + diamide	10/5	0.38 ± 0.40	nd

[a] The prothrombinase activity is expressed per packed cell volume.

the inner leaflet in cells treated with NEM. Similar results were found in cells incubated at 25 °C.

Figure 4B gives the equilibrium level for the redistribution of spPS after 1 h incubation at 25 °C. While most of the spPS is translocated to the inner monolayer in control cells, a significant amount of spPS is still present in the outer monolayer of oxidatively damaged cells. These data indicate that various levels of oxidative stress can affect the flipase activity to a different extent. Since the flipase retained activity under a number of conditions, it could account for the lack of PS in the outer monolayer as determined by annexin labeling or prothrombinase activation as indicated above. To investigate whether an impaired flipase activity under those conditions would lead to the formation of an annexin binding population, cells were pretreated with 10 mM NEM (which blocks flipase activity) prior to incubation with hydrogen peroxide. As shown in Table 2, this combined treatment did not result in enhanced annexin labeling of the cells. Since diamide was reported to increase PC movement across the bilayer (Franck et al., 1986), and loss of phospholipid asymmetry could be expected, cells were also incubated with NEM prior to diamide treatment. Similar results as compared to treatment with hydrogen peroxide were found (Table 2), suggesting that the lack of exposure of PS under these conditions was not due to an active restoration of phospholipid asymmetry by flipase. Rather, an absence of movement from the inner to the outer monolayer seemed to be responsible for the absence of PS in the outer monolayer.

These data taken together show that in none of the investigated cases of oxidation, neither by separate treatment with NEM, diamide, phenylhydrazine, hydrogen peroxide (in combination with azide), *tert*-butyl hydroperoxide, or cumene hydroperoxide, nor by combined treatments of NEM and diamide or hydrogen peroxide, could any significant loss of asymmetry be observed.

While oxidants do not lead to the exposure of PS, an increase in cytosolic calcium is very effective in scrambling of the bilayer (Williamson et al., 1992; Kuypers et al., 1996a). An increase in cytosolic calcium will lead to a progressive increase in the PS-exposing subpopulation in time (Kuypers et al., 1996a). After 1 h, approximately 50% of the cells expose PS when incubated at 16% hematocrit in 1 mM calcium and 4 μM A23187 (Figure 5). These data could suggest that in the other 50% of the population, an increased PS movement from the inner to the outer monolayer is counteracted by an active flipase. This seems confirmed by the increased number of cells that expose PS after inhibition of the flipase by NEM (Figure 5). Incubation

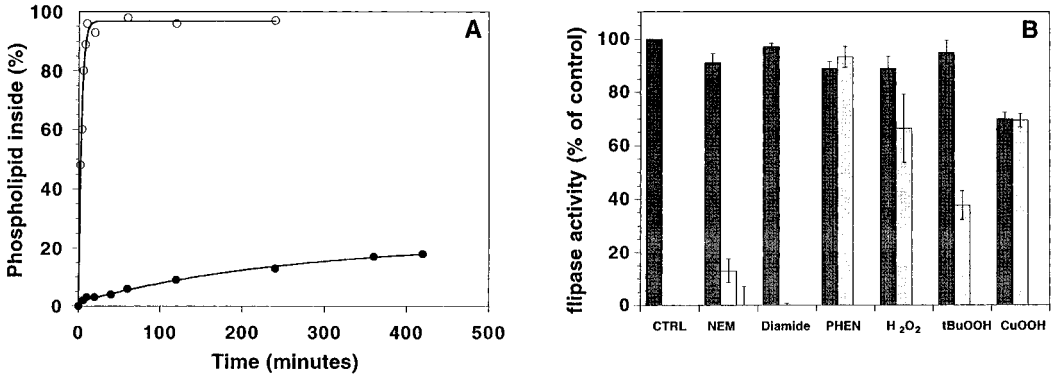

FIGURE 4: Determination of flipase activity in sulfhydryl-modified and oxidized cells. (A) Effect of NEM treatment on transbilayer movement of spin-labeled PS. Erythrocytes were incubated in the absence (open symbols) or presence (closed symbols) of 5 mM NEM for 10 min at 37 °C, washed, and labeled with spPS. The amount of label in the outer monolayer was determined by back-exchange to BSA as described under Materials and Methods. The results are expressed as the percentage of the total spPS present in the inner monolayer (nonextractable by BSA). The curves are fitted to the experimental data by least-squares exponential nonlinear regression. (B) Percentage of flipase activity remaining in cells treated with 0.5 (dark bars), 5 (light bars), or 10 mM NEM; 0.5 (dark bars) or 5 mM (light bars) diamide, phenylhydrazine (PHEN), hydrogen peroxide (H_2O_2), or *tert*-butyl hydroperoxide (tBuOOH); and 0.5 (dark bar) or 1 mM (light bar) cumene hydroperoxide (CuOOH), expressed relative to flipase activity in control cells (CTRL). Data are the average and standard deviation of two experiments.

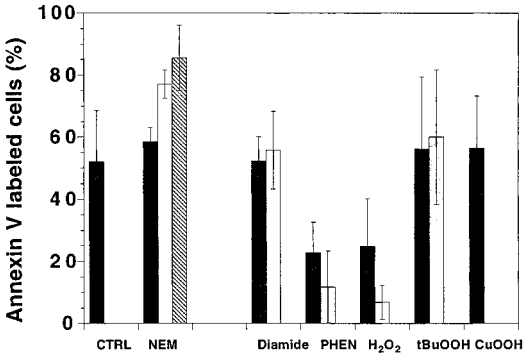

FIGURE 5: Binding of FITC−annexin V to sulfhydryl-modified or oxidized cells treated with calcium and ionophore. Erythrocytes were incubated for 1 h at 37 °C at a hematocrit of 40% in the absence (CTRL) and presence of 0.5, 5, or 10 mM NEM; 0.5 or 5 mM diamide, phenylhydrazine (PHEN), hydrogen peroxide (H_2O_2), or *tert*-butyl hydroperoxide (tBuOOH); or 0.5 mM cumene hydroperoxide (CuOOH). Subsequently cells were treated with 1 mM calcium in the presence of 4 μM ionophore A23187 and labeled with annexin V as described under Materials and Methods. 0.5 mM, dark bars; 5 mM, light bars; 10 mM, hatched bars. The data indicate the average and standard deviation of two to four independent experiments.

with NEM prior to treatment with calcium and ionophore enhanced the scrambling normally induced by this treatment in a concentration-dependent way, as can be concluded from the increase in annexin binding fraction of the cells.

In contrast, diamide pretreatment, which also severely inhibited the flipase (Figure 4B), did not significantly increase the calcium- and ionophore-induced scrambling (Figure 5), suggesting that diamide also affected the calcium-induced movement of PS from the inner to the outer leaflet.

Incubation in the presence of phenylhydrazine, hydrogen peroxide, *tert*-butyl hydroperoxide, and cumene hydroperoxide did not, or only partly, inhibit the flipase activity (Figure 4B). Treatment with *tert*-butyl hydroperoxide or cumene hydroperoxide prior to treatment with calcium and ionophore did not increase the population of annexin-labeled cells significantly (Figure 5), suggesting that the flipase was able to restore in part the calcium-induced scrambling as in nonoxidized control cells. In contrast, annexin labeling was reduced after cells were incubated with hydrogen peroxide or phenylhydrazine prior to calcium and ionophore treatment, indicating a decreased ability for calcium to scramble phospholipids in these cells.

The latter result was confirmed with the prothrombinase assay. When control cells or cells previously oxidized with NEM, diamide, *tert*-butyl hydroperoxide, and cumene hydroperoxide were treated with calcium and ionophore, the prothrombinase activity in the presence of these cells was 40 $\pm$ 11 units/μL thrombin per minute per packed cell equivalent, which is approximately 80% of the activity obtained in the presence of completely hemolyzed cells. However, cells pretreated with hydrogen peroxide or phenylhydrazine induced a prothrombinase activity of only 9.3 $\pm$ 3.3 units/μL thrombin per minute per packed cell volume, approximately 23% of the value in the presence of other oxidants or control cells treated similarly.

Although these results strongly suggest that the calcium-induced scrambling was impaired in these cells, it was needed to verify that calcium was still able to enter cells pretreated with oxidants. To obtain scrambling, it is necessary to significantly raise the intracellular calcium concentration. In the case of oxidative damage, ion channels might not be efficiently formed upon treatment with ionophore A23187. Therefore, cells that had been oxidized with 5 mM diamide or hydrogen peroxide were loaded with the fluorescent calcium indicator Fluo-3, then treated with calcium and ionophore, and subsequently analyzed for the increase in calcium by flow cytometry as described under Materials and Methods. Oxidative damage resulted in the formation of fluorescent products, as indicated in Figure 1B. The increased fluorescence as observed after Fluo-3 loading appeared superimposed upon this autofluorescence (Figure 6). Calcium internalization was visualized by a strong shift in fluorescence as compared to the Fluo-3-loaded cells. The observed intensity shifts in Fluo-3 fluorescence (Figure 6) show that Fluo-3 loading was possible in erythrocytes oxidized with diamide and hydrogen peroxide, and that calcium was able to enter oxidized cells during treatment with ionophore. Hence, the calcium concentration after ionophore treatment had increased in oxidized cells similar to previously untreated cells. In contrast to normal cells, however, this increase in calcium did not as efficiently lead to PS exposure. These data indicate a similar A23187-

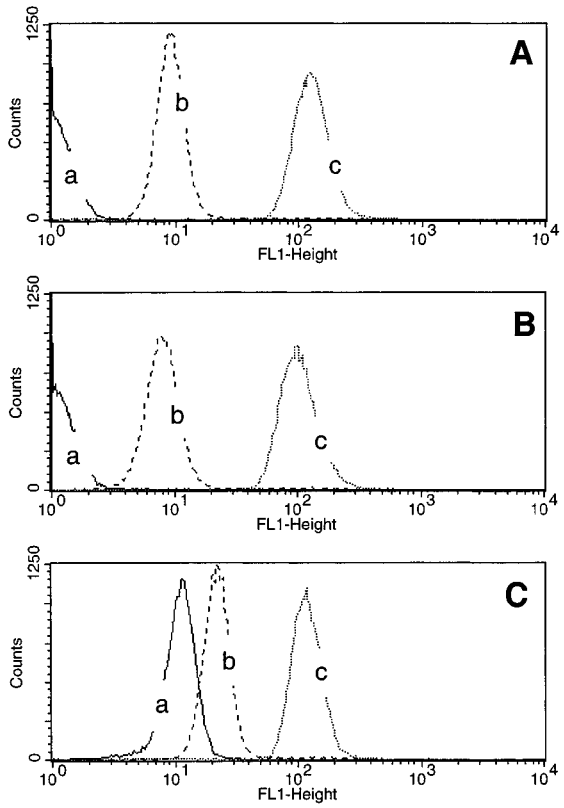

FIGURE 6: Flow cytometric analysis of calcium internalization. Erythrocytes were loaded with the calcium indicator Fluo-3 and treated with calcium and ionophore A23187 as described under Materials and Methods. Plots indicate fluorescence intensities of erythrocytes before (curve a) and after (curve b) loading with Fluo-3, and after subsequent treatment of the cells with calcium and ionophore in the presence of intracellular Fluo-3 (curve c). (A) Control erythrocytes. (B) Erythrocytes pretreated with 5 mM diamide at 40% hematocrit for 1 h at 37 °C. (C) Erythrocytes pretreated with 5 mM hydrogen peroxide at 40% hematocrit for 1 h at 37 °C.

induced calcium uptake in all cells regardless of treatment. Oxidation, however, can lead to a decrease in the population of cells that are scrambled by calcium. Again, an active flipase might counteract the loss of the phospholipid asymmetry in these cells. To investigate this possibility, the flipase activity was inhibited by pretreatment with 10 mM NEM prior to incubation with 5 mM hydrogen peroxide. Subsequently the cells were subjected to treatment with calcium and ionophore and labeled with annexin V. An increase in the population of cells that expose PS from 5% (Figure 5) to 40% was observed as compared to cells not treated with NEM. The presence of a subpopulation of cells that were not labeled with annexin under these conditions indicates that hydrogen peroxide inhibits the mechanism of calcium-induced scrambling.

DISCUSSION

In the past several years, oxidative damage to erythrocytes has become a keyword for the explanation of destructive events in many hematological disorders or in aged cells. In numerous reports, the suggestion has been made that oxidative stress would, directly or indirectly, be the factor responsible for membrane alterations (Jain, 1985; Jain & Shohet, 1984; Shinar & Rachmilewitz, 1990; Wolfe et al., 1989; Kuypers et al., 1996b), that would ultimately cause the observed physiological events, such as imbalanced hemostasis and vaso-occlusive crisis in sickle cell disease or removal of the cells from the bloodstream in thalassemia. While PS is found entirely in the cytoplasmic monolayer in normal erythrocytes (Op den Kamp, 1979; De Jong et al., 1996; Kuypers et al., 1996), in many cases that might involve higher levels of oxidative stress, exposure of PS on the membrane surface has been detected (Tait & Gibson, 1994; Kuypers et al., 1996a; Borenstain-Ben Yashar, 1993; Yuan et al., 1994; Connor et al.; 1994; Wilson et al., 1993). Cells exhibit a procoagulant surface when PS is exposed (Zwaal et al., 1992), and loss of PS asymmetry has been demonstrated to play a role in recognition of cells by macrophages, which has led to the hypothesis that PS can serve as a signal for removal of defective cells from the bloodstream (Fadok et al., 1992; McEvoy et al., 1986; Connor et al., 1994).

The apparent connection between oxidative damage and disturbance of PS asymmetry has led us to investigate a relationship between these events. Erythrocytes were treated with low (0.5 mM) or high (5 or 10 mM) concentrations of either NEM, diamide, phenylhydrazine, hydrogen peroxide, *tert*-butyl hydroperoxide, and cumene hydroperoxide or combinations of these to provide a broad spectrum of types of sulfhydryl modification or oxidative damage. The oxidative damage, induced by the use of these chemical reagents in this study, exceeds loss of parameters of cellular competence as compared to those reported for most red cell pathologies such as thalassemia and sickle cell anemia.

We measured PS exposure using two techniques that have been proven to provide very sensitive and noninvasive tools to measure specifically small changes in PS asymmetry in membrane systems: labeling with FITC-conjugated annexin V (Kuypers et al., 1996a) and the prothrombinase assay (Bevers et al., 1982; De Jong et al., 1996). The use of FITC-AV allows the analysis of individual cells and has this advantage over traditional methods, that analyze the population as a whole. The appearance of PS-exposing cells in a population can be determined by the PS-dependent prothrombinase assay, and our data indicate (Figure 3) that an increase in prothrombinase activity in a cell population can be interpreted as the exposure of PS in a subpopulation of cells while the rest of the population exhibits a normal phospholipid asymmetry.

We observed neither significant exposure of PS in the population as a whole nor the appearance of a subpopulation of cells that have lost their phospholipid asymmetry as the result of sulfhydryl modification or oxidation (Table 2). For the exposure of PS on the outer surface, an inhibition of PS movement from the outer to the inner monolayer seems required in addition to an increased movement of PS from the inner to the outer monolayer. Active restoration of PS asymmetry by the flipase would counteract the exposure of PS and give negative prothrombinase or annexin binding results. Sulfhydryl modification has been reported to reduce the activity of the flipase (Morrot et al., 1989; Herrmann & Devaux, 1990), and our data also indicate that NEM effectively inhibits flipase activity (Figure 4A). Combined treatment of erythrocytes with NEM and other oxidants did not result in an increase in annexin V labeled cells either (Table 2), indicating that the flipase did not counteract potential oxidant-induced loss of membrane asymmetry.

It can be concluded that sulfhydryl modification or oxidant damage induced by the compounds used will not be the direct cause of PS asymmetry loss in erythrocytes. Increase of the

intracellular calcium concentration to millimolar levels, on the other hand, seems to efficiently expose PS on the outer surface of the bilayer (Williamson et al., 1992). The mechanism for this membrane scrambling is not completely clear. While it was reported that PIP_2 played a key role in this calcium-induced scrambling (Sulpice et al., 1994, 1996), this was disputed in other reports (Bevers et al., 1996), which indicated the involvement of a specific protein, the scramblase (Bassé et al., 1996).

Whether a specific scramblase (Bevers et al., 1996; Bassé et al., 1996) and/or PIP_2 (Sulpice et al., 1994, 1996) is involved in the calcium-induced scrambling of the bilayer, the process pivots around the availability of ATP. An increase in cytosolic calcium, and calcium-induced phospholipid scrambling, will be counteracted by the ATP-consuming outward-directed calcium pump, and the ATP-dependent flipase which will relocate PS to the inner monolayer.

It can be expected that those cells in the population that have lost the ability to transport PS and calcium will be labeled with annexin V. The gradual increase in time of the number of cells that expose PS as the result of calcium- and ionophore-induced membrane scrambling seems to confirm this interpretation (Kuypers et al., 1996a; Williamson et al., 1992). Oxidants or sulfhydryl reagents can interfere at different levels in this calcium-induced scrambling process, and affect the movement from the inner to the outer monolayer as well as the active transport from the outer to the inner leaflet by the flipase.

Our data show that different reagents will act differently on the flipase activity. While the sulfhydryl reagents NEM and diamide efficiently inhibit the flipase, this is not the case for the oxidative agents used (hydrogen peroxide, phenylhydrazine, *tert*-butyl hydroperoxide, cumene hydroperoxide) even under conditions that severely affect the integrity of the cell.

Our data also show that different reagents act differently on the level of calcium-induced membrane scrambling. Upon treatment with calcium and ionophore under our conditions, the number of nonoxidized control cells that expose PS gradually increases in time, and after 1 h approximately 50% of the cells label with FITC-AV (Figure 5). This suggests that the flipase is active and able to restore asymmetry in the other half of the cells. Pretreatment with the oxidants *tert*-butyl hydroperoxide and cumene hydroperoxide does not affect the flipase significantly, and also does not affect the calcium-induced PS exposure, which is reflected by the similar level of scrambling as found in nonoxidized calcium- and ionophore-treated cells. These data indicate that they have no major role in the processes that would lead to calcium-induced PS exposure. Inhibition of the flipase would lead to more cells that expose PS upon calcium-induced scrambling since restoration of phospholipid asymmetry cannot take place, which is indeed found in the case of NEM pretreatment. Diamide pretreatment, on the other hand, although efficient in the inhibition of the flipase, does not increase the number of cells that expose PS following treatment with calcium and ionophore, and therefore seems to interfere with the calcium-induced scrambling process also. Moreover, hydrogen peroxide and phenylhydrazine do not affect the flipase effectively but reduce the calcium-induced PS exposure, indicating that they inhibit the calcium-induced scrambling process. After incubation with phenylhydrazine or hydrogen peroxide (in the presence of azide) and subsequent treatment with calcium and ionophore, the fraction of annexin-labeled cells did not exceed 25% (Figure 5). Flipase inhibition by NEM treatment, prior to oxidation with hydrogen peroxide, increased subsequent calcium-induced scrambling to 40% of the population, compared to 95% after treatment with NEM only prior to calcium-induced membrane scrambling. This seems to exclude the possibility that the increased activity of the flipase in hydrogen peroxide treated cells accounted for the observed decrease in annexin V labeled cells. In contrast, this result indicates that the calcium-induced scrambling is less efficient after oxidation with hydrogen peroxide.

Our data do not specify how these agents affect this scrambling process. However, it is not caused simply by interfering with the A23187-induced calcium increase in the cytosol. In all cases, calcium was still able to enter the cells as shown by loading of the cells with the calcium indicator Fluo-3 (Figure 6).

The exact mechanism of phospholipid scrambling has not been resolved to date, and based on our data, we can only speculate as to what caused this observed inhibition of calcium-induced membrane scrambling. The profound differences found for the different oxidants might be related to their site of action. While the lipid bilayer is the major target for oxidants such as cumene hydroperoxide, hydrogen peroxide and phenylhydrazine cause more damage in the cytosol, in particular to hemoglobin (Van den Berg et al., 1992; Snyder et al., 1986). This suggests that modified cytosolic components such as oxidized hemoglobin interfere with processes in the plasma membrane that induce loss of phospholipid asymmetry. Oxidative damage of hemoglobin has been shown to affect the red cell membrane by interaction with spectrin (Snyder et al., 1986) or PS in the bilayer (Liu et al., 1996; Brunauer et al., 1994; Moxness et al., 1996).

In conclusion, our study shows that sulfhydryl modification or oxidation of erythrocytes does not directly lead to exposure of PS, even under conditions that severely damage the cell and inhibit flipase activity. Moreover, certain types of sulfhydryl modifications or oxidative damage can interfere with calcium-induced scrambling of the bilayer. Since the oxidative damage induced by several conditions in this study exceeds the loss of similar parameters of cellular competence in pathologic red cells, it is unlikely that the loss of phospholipid asymmetry that is observed in thalassemia and sickle cell anemia (Tait & Gibson, 1994; Kuypers et al., 1996a; Borenstain-Ben Yashar et al., 1993; Yuan et al., 1994) is simply due to increased levels of oxidative stress.

Hence, other factors seem to be involved in those cases in which increased oxidative damage was implicated in the exposure of PS on the surface of pathologic red cells.

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