Generation of Singlet Oxygen Induces Phospholipid Scrambling in Human Erythrocytes

P. M. Hilarius, I. G. Ebbing, D. W. C. Dekkers, J. W. M. Lagerberg, D. de Korte, and A. J. Verhoeven*

Sanquin Research at CLB, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

Received September 11, 2003; Revised Manuscript Received February 2, 2004

ABSTRACT: Maintenance of phospholipid asymmetry of the plasma membrane is essential for cells to prevent phagocytic removal or acceleration of coagulation. Photodynamic treatment (PDT), which relies on the generation of reactive oxygen species to achieve inactivation of pathogens, might be a promising approach in the future for decontamination of red blood cell concentrates. To investigate whether PDT affects phospholipid asymmetry, erythrocytes were illuminated in the presence of 1,9-dimethyl-methylene blue (DMMB) as photosensitizer and subsequently labeled with FITC-labeled annexin V. This treatment resulted in about 10% annexin V positive cells, indicating exposure of phosphatidylserine (PS). Treatment of erythrocytes with *N*-ethylmaleimide (NEM) prior to illumination, to inhibit inward translocation of PS via the aminophospholipid translocase, resulted in enhanced PS exposure, while treatment with H_2O_2 (previously shown to inhibit phospholipid scrambling) greatly diminished PS exposure, indicating the induction of phospholipid scrambling by PDT. Only erythrocytes illuminated in the presence of DMMB showed translocation of NBD-phosphatidylcholine (NBD-PC), confirming scrambling induction. Double label experiments indicated that PS exposure does not occur without concurrent scrambling activity. Induction of scrambling was only moderately affected by Ca^{2+} depletion of the cells. In contrast, scavengers of singlet oxygen were found to prevent phospholipid scrambling induced by PDT. The results of this study show that phospholipid scrambling is induced in human erythrocytes by exposure to singlet oxygen.

Photodynamic treatment (PDT)[1] might be a promising approach in the future for pathogen inactivation in erythrocyte concentrates destined for transfusion. Photodynamic treatment relies on the use of light-sensitive compounds (photosensitizers) which have an affinity for DNA (and RNA) and which, upon illumination, form reactive oxygen species (ROS). The ROS formed inactivate pathogens but may also induce damage to red blood cell components (*1*, *2*). Since the maintenance of phospholipid asymmetry is essential for cell survival and prevention of procoagulant activity, a successful procedure for PDT of erythrocytes should not only leave the integrity of the plasma membrane intact but also be without effect on the phospholipid asymmetry of the plasma membrane.

Under normal conditions, erythrocytes exhibit an asymmetric distribution of phospholipids in the membrane (*3*). The choline-containing lipids, phosphatidylcholine (PC) and sphingomyelin, predominantly reside in the outer leaflet, while the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are found mainly in the inner leaflet (*4*). When PS or PE appears on the outer leaflet, the ATP-dependent aminophospholipid translocase restores the normal, asymmetric phospholipid distribution. Another transporter (multidrug resistance protein 1, MRP1) has been suggested to play a role in outward movement of phospholipids (*5*, *6*), and these activities, together with as yet unknown transporters, may constitute a continuous cycle of translocation (*3*). In freshly isolated erythrocytes, PS exposure can be detected in only 0.2% of the total population by means of annexin V−FITC binding (*7*). Boas et al. (*8*) have shown that increased PS exposure does occur during normal red blood cell aging in vivo and plays a role in sequestration of senescent erythrocytes. In vitro, severe loss of phospholipid asymmetry can be induced by treatment of erythrocytes with Ca^{2+} and Ca^{2+} ionophore (*9*), and this has been ascribed to activation of a phospholipid scramblase (*10*). Phospholipid scramblase catalyzes the bidirectional movement of phospholipids across the membrane, showing no phospholipid specificity (*11*).

In this report it is shown that phospholipid scrambling can be induced not only by elevation of intracellular Ca^{2+} but also by exposure to singlet oxygen generated during PDT of erythrocytes.

MATERIALS AND METHODS

1,9-Dimethyl-methylene blue (DMMB) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were obtained from Aldrich (Steinheim, Germany). *N*-Ethylmaleimide (NEM), dipyridamole (DIP), and indomethacin were from Sigma (St. Louis, MO). Perhydrol (H_2O_2) and sodium

* Address correspondence/reprint requests to this author. Telephone: 31-20-5123317. Fax: 31-20-5123310. E-mail: a.verhoeven@sanquin.nl.

[1] Abbreviations: BAPTA, *N*,*N*-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]]glycine bis[(acetyloxy)methyl] ester; BSA, bovine serum albumin; DMMB, 1,9-dimethyl-methylene blue; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FITC, fluorescein isothiocyanate; GAM, goat anti-mouse IgG; MRP1, multidrug resistance protein 1; NBD, 6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PDT, photodynamic treatment; PE, phosphatidylethanolamine; PLSCR-1, phospholipid scramblase 1; PS, phosphatidylserine; ROS, reactive oxygen species; TLC, thin-layer chromatography.

10.1021/bi035637k CCC: $27.50
Published on Web 03/13/2004

azide were purchased from Merck (Darmstadt, Germany). Annexin V−FITC was from VPS-Diagnostics (Hoeven, The Netherlands). Ionophore A23187, BAPTA-AM, and Fluo-3-AM were obtained from Molecular Probes (Eugene, OR). Annexin V−Cy5 was from Becton and Dickinson. 1-Palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoserine (NBD-PS) and 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were from Avanti Polar Lipids (Alabaster, AL).

Isolation of Erythrocytes. Leukocyte-reduced erythrocytes were prepared by centrifugation of whole blood collections (8 min, 2300*g*) and subsequent removal of the buffy coat (*12*, *13*), followed by filtration of the erythrocyte suspension to remove residual leukocytes. The resulting suspensions had a volume of 250−300 mL, a Ht of about 60%, and contained $<1 \times 10^6$ leukocytes per unit (as determined with a Nageotte hemocytometer), whereas platelet counts were below detection limit (determined with an AcT10; Coulter Electronics Ltd., Dunstable, U.K.). Erythrocyte filtration and all further manipulations were performed at a temperature of 22 ± 2 °C.

Photodynamic Treatment (PDT). Erythrocyte suspensions were washed twice in incubation medium consisting of 134 mM NaCl, 10 mM glucose, 10 mM Tris, pH 7.4, and 40 mM sucrose (SGT/sucrose). Washed cell suspensions (diluted to a Ht of 30%) were incubated for 10 min at room temperature with 0−40 μM DMMB as photosensitizer, after which they were placed in polystyrene (48 flat-bottom well) cell culture plates (Corning Inc., Corning, NY; 300 μL of erythrocyte suspension per well) and illuminated.

Phototreatment was performed in a setup that was developed in house (*14*), consisting of a halogen lamp (Philips, 300 W), a cutoff filter (RG610, transmitting light at $\lambda >$ 560 nm) (Schott, Mainz, Germany), and a rocking platform. During illumination, plates were placed on a 1,4-butanediol cooling plate in order to maintain the temperature of the irradiated cell suspension at 22 °C. Furthermore, care was taken to eliminate a substantial part of the infrared radiation by interposition of a vacuum glass plate and a water bath between the lamp and the cells. Fluency was 30 mW/cm^2 as measured with a IL1400A photometer, equipped with a SEL033/F/U detector (International Light, Newburyport, MA). The illumination time was 20 min, corresponding to a light dose of 360 kJ/m^2.

Hemolysis. Hemolysis of the different erythrocyte suspensions was determined by measurement of the Hb content of cell supernatants by the Hb cyan method (*15*). Cell supernatants were obtained by centrifugation of the cell suspensions for 15 s at 12000*g*. Hemolysis was expressed as a percentage of total hemoglobin present in erythrocyte lysates.

Annexin V Labeling of Erythrocytes. To quantify the amount of erythrocytes exposing PS on their cell surface, cells were washed once after phototreatment and stained with FITC-labeled annexin V essentially following the method of Kuypers et al. (*7*). Cells were washed in SGT/sucrose, supplemented with 20 mM Hepes and 0.5% HSA (final pH 7.4), and resuspended at 0.3% Ht in the same medium supplemented with 2.5 mM $CaCl_2$. Labeling with annexin V was performed by adding annexin V−FITC (final concentration 1 μg/mL) to 250 μL of cell suspension. After incubation at room temperature in the dark for 30 min, cells were washed once and analyzed on a Becton Dickinson FACScan flow cytometer. Data analysis was performed using the WinMDI 2.8 software program (Scripps Research Institute, La Jolla, CA). The percentage of annexin V positive cells was determined by comparison with a negative control incubated with GAM−FITC (1 μg/mL), which was run for each sample in parallel to account for autofluorescence changes after some of the (pre)treatments (see below).

Pretreatment of Erythrocytes To Modulate Phospholipid Transport Activities. Freshly isolated erythrocytes were washed, resuspended in PBS at 40% Ht, and incubated for 1 h at 37 °C in the presence of 10 mM NEM or 5 mM H_2O_2 (in combination with 4 mM sodium azide to inhibit catalase activity) according to de Jong et al. (*10*). After the preincubation, a sample was taken to determine the amount of hemolysis, and the cells were washed in PBS, followed by washing and resuspension in SGT/sucrose. Cells were subsequently illuminated and analyzed for annexin V binding as described above. Control experiments indicated that when the cells, instead of being illuminated, were exposed to 1 mM $CaCl_2$ in combination with 5 μM A23187, NEM pretreatment enhanced annexin V binding 2-fold, whereas H_2O_2 pretreatment resulted in an almost complete inhibition, in accordance with de Jong et al. (*10*).

Translocation of NBD-phospholipids. Immediately after illumination, cells were diluted to 5% Ht (equivalent with 5 $\times 10^8$ cells/mL) and incubated for 5 min at 37 °C in a volume of 0.5 mL. The assay was started by the addition of 5 μL of NBD-phospholipid (200 μM; final concentration 2 μM). These stock solutions of NBD-PC or NBD-PS were freshly prepared by drying a stock solution in $CHCl_3$ and subsequent resuspension in SGT/sucrose. At different time intervals, 20 μL aliquots were removed from the cell incubations and diluted in 1 mL of ice-cold SGT/sucrose with 1% BSA to extract label present in the outer leaflet of the membrane (*16*). For measuring total fluorescence, samples were mixed with SGT/sucrose in the absence of BSA. After at least 5 min extraction time, samples were introduced into the flow cytometer, measuring forward scatter, side scatter, and green fluorescence. The data were analyzed using the WinMDI 2.8 software program. The fraction of probe translocated was determined by dividing the mean fluorescence of the sample population after BSA extraction (internalized probe) by the mean fluorescence of the same population in the absence of BSA (total probe bound). Analysis of the cell supernatant indicated that virtually all of the label added was bound by the cells. The integrity of the NBD probes was verified with TLC. No degradation of the probes was observed during the course of the experiments.

In some experiments, NBD-PC translocation was measured in parallel with detection of annexin V binding. For this, cells were illuminated in the presence of 20 μM DMMB and subsequently diluted to 0.3% Ht and incubated for 5 min at 37 °C in the presence of 2.5 mM $CaCl_2$ in a volume of 500 μL. The assay was started by the addition of NBD-PC (final concentration 0.12 μM) and annexin V−Cy5 (80 ng/mL). The lower concentration of NBD-PC of 0.12 μM was used instead of 2 μM to maintain the ratio of NBD-lipid to cells at this lower hematocrit (0.3% vs 5%). Samples were processed as described above and analyzed for NBD-PC fluorescence (FL1) and annexin V−Cy5 fluorescence

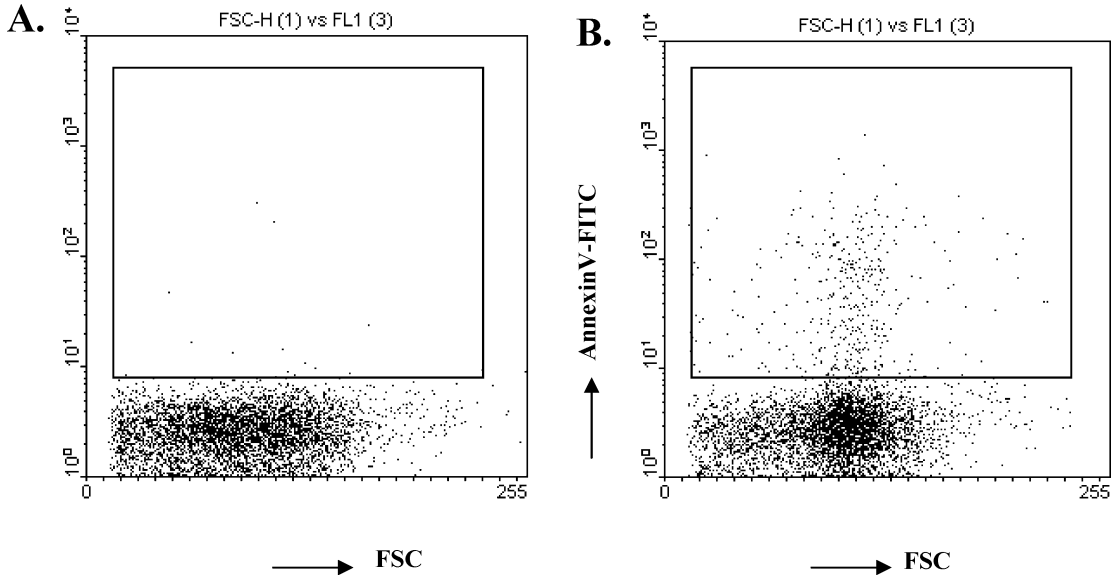

FIGURE 1: Induction of annexin V binding to erythrocytes by photodynamic treatment. Erythrocytes (Ht 30%) were either untreated (A) or illuminated with 360 kJ/m^2 in the presence of 20 μM DMMB (B) and labeled with annexin V as described under Materials and Methods. The figures are representative dot plots of forward scatter (FSC) against green fluorescence (annexin V−FITC).

(FL4) in a FACSCalibur (Becton and Dickinson) flow cytometer.

Ca^{2+} Depletion of Erythrocytes. To investigate the role of changes in intracellular free Ca^{2+}, erythrocytes (Ht 0.3%) were loaded with BAPTA by preincubation at 37 °C for 45 min in SGT/sucrose in the presence of 10 μM BAPTA/AM. In part of the cells, BAPTA loading was accompanied by Fluo-3 loading by the addition of 1 μM Fluo-3/AM to enable measurement of intracellular free Ca^{2+} changes (*10*). After two washing steps, Fluo-3-loaded cells (Ht 3%) were analyzed in a Becton Dickinson FACScan flow cytometer. BAPTA-loaded cells and control cells (Ht of 3%) were subsequently treated with 20 μM DMMB and a light dose of 75 kJ/m^2. The lower light dose was chosen to induce comparable PS exposure as induced by the treatment of an erythrocyte suspension of 30% Ht used in most other experiments. Annexin V binding and NBD-PC translocation were measured separately as described above, except that the Ht was 3% during NBD-PC uptake for which 1.2 μM NBD-PC was added instead of 2 μM. In the case of BAPTA-loaded cells, PDT and measurement of PS exposure and NBD-PC translocation were performed in the presence of 2.5 mM EGTA to ensure complete Ca^{2+} depletion.

RESULTS

In accordance with literature data (*7*), untreated erythrocytes hardly showed any annexin V binding (Figure 1A), indicating that PS was localized to the inner leaflet of the red cell membrane. However, illumination of erythrocytes in the presence of DMMB as photosensitizer induced a significant number of cells to bind annexin V (Figure 1B). Cells illuminated in the absence of DMMB or cells incubated with DMMB without subsequent illumination did not show annexin V binding (data not shown). Although some changes in FSC distribution were noted after PDT (Figure 1B), annexin V binding was observed throughout the FCS distribution pattern. The extent of the PDT-induced PS exposure was dependent on both sensitizer concentration (Figure 2) and light dose (data not shown).

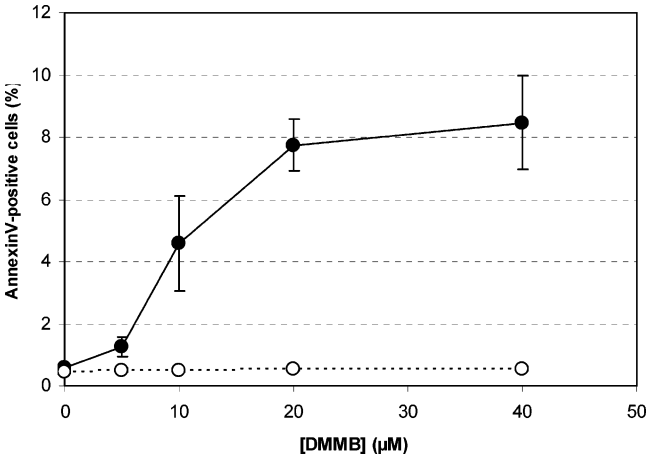

FIGURE 2: Dependence of PS exposure of illuminated erythrocytes on DMMB concentration. Erythrocytes (Ht 30%) were incubated with different DMMB concentrations and subsequently illuminated with 360 kJ/m^2 (closed symbols) or left in the dark (open symbols). Samples were incubated with annexin V−FITC as described under Materials and Methods. Results shown are the mean (±SEM) of four experiments.

It should be noted that, in the experiments described above, 40 mM sucrose was present to prevent hemolysis induced by PDT (*17*). Under these conditions, there was no induction of hemolysis, even not when the cells, following illumination, were incubated at 37 °C (data not shown). Incubation at 37 °C for 60 min following illumination did increase the percentage of annexin V positive cells (from 12.3 ± 0.7 to 31.0 ± 5.5, mean ± SEM of four experiments), indicating that the PS exposure induced by PDT is enhanced rather than reversed by incubation at 37 °C.

To study the underlying mechanism of the loss of phospholipid asymmetry induced by PDT, erythrocytes were pretreated with various inhibitors of phospholipid transport. According to de Jong et al. (*10*), the sulfhydryl-modifying compound NEM inhibits the aminophospholipid translocase, while the oxidative agent H_2O_2 inhibits phospholipid scrambling, as was shown in erythrocytes treated with Ca^{2+} and

Table 1: Effect of Phospholipid Transport Inhibitors on PS Exposure after PDT[a]

addition	PS-positive cells (%)
none	7.7 ± 0.5
+NEM	24.2 ± 1.1
$+H_2O_2$	0.2 ± 0.1
+indomethacin	8.3 ± 0.9

[a] Erythrocytes were pretreated with NEM or H_2O_2 as described under Materials and Methods. Indomethacin (300 μM) was added to erythrocytes (Ht 30%) and incubated for 30 min at room temperature. Cells were subsequently illuminated (Ht 30%, 360 kJ/m^2, 20 μM DMMB) and analyzed for PS exposure by annexin V labeling. Results shown are the mean (±SEM) of four experiments. Both NEM and H_2O_2 treatment resulted in a significant difference as compared to the control ($P < 0.0005$, Student's *t*-test for paired observations), whereas indomethacin had no significant effect ($P > 0.05$).

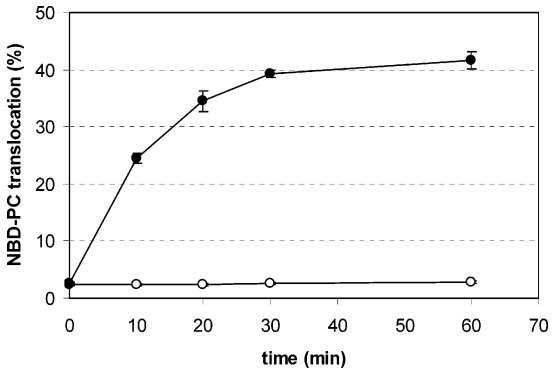

FIGURE 3: Photodynamic treatment induces inward translocation of NBD-PC. Control and illuminated erythrocytes (Ht 30%, 360 kJ/m^2, 20 μM DMMB) were diluted to 5% Ht and incubated with 2 μM NBD-PC at 37 °C. At different time intervals, samples were analyzed for the amount of label translocated to the inner leaflet as described under Materials and Methods. Key: (●) illuminated; (○) control. Results shown are the mean (±SEM) of four experiments.

Ca^{2+} ionophore. Indomethacin was used as an inhibitor of MRP1 (*5*). Treatment of erythrocytes with NEM prior to illumination resulted in an enhanced PS exposure (Table 1) while treatment with H_2O_2 greatly diminished PS exposure. Indomethacin added at a concentration well above its K_i for MRP1 inhibition (*5*) showed no effect. The pretreatment of the cells with NEM by itself did not induce PS exposure (data not shown). These results suggested that, similar to the effect of Ca^{2+} and Ca^{2+} ionophore, PS exposure of erythrocytes after PDT is induced by induction of scrambling activity.

To confirm scrambling activity after PDT, we subsequently measured the inward translocation of NBD-PC. In erythrocytes, NBD-PC is internalized at a very low rate, unless the cells are activated with Ca^{2+} and Ca^{2+} ionophore (*18*). Using flow cytometry (*16*), we confirmed this observation (data not shown). When control and photodynamically treated erythrocytes were incubated with NBD-PC at 37 °C, only the illuminated cells showed inward translocation of the fluorescent lipid over a 60 min incubation period (Figure 3), indicating scrambling activity. In support of this, we observed that in untreated erythrocytes translocation of NBD-PS was completely prevented by NEM pretreatment (indicating the involvement of aminophospholipid translocase in this process), whereas in erythrocytes subjected to PDT translocation of NBD-PS could still be observed after NEM pretreatment (Figure 4), indicating the involvement of another transport system.

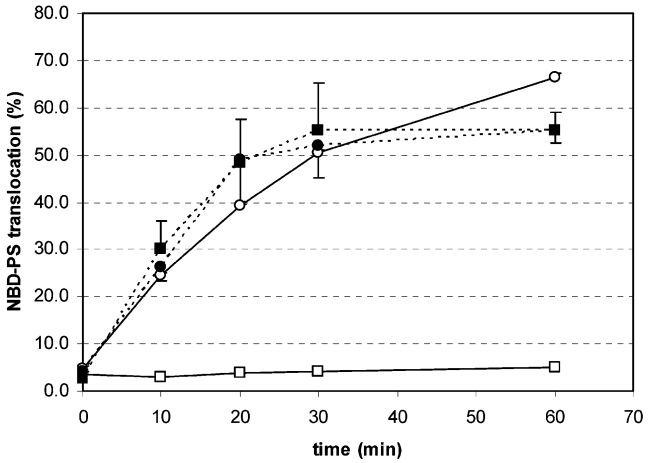

FIGURE 4: Effect of NEM on NBD-PS translocation. Erythrocytes were pretreated with NEM as described under Materials and Methods. Control cells were preincubated without NEM. Part of these cells were illuminated under standard conditions (Ht 30%, 360 kJ/m^2, 20 μM DMMB), while another part was left untreated. Subsequently, all suspensions were diluted to 5% Ht and incubated with 2 μM NBD-PS at 37 °C. At different time intervals, samples were analyzed for the amount of label translocated to the inner leaflet as described under Materials and Methods. Key: (○) without NEM, no DMMB treatment; (□) with NEM, no DMMB treatment; (●) without NEM, DMMB-treated; (■) with NEM, DMMB-treated. Results shown are the mean (±SEM) of three experiments.

To establish the relationship between phospholipid scrambling (as indicated by NBD-PC translocation) and PS exposure (as indicated by annexin V binding), double labeling experiments were performed. Under these conditions, no PS-positive cells could be detected at the start of these incubations despite PDT (Figure 5A, upper left), probably due to the immediate dilution of both cells and label in cold buffer at zero time. In the first 10 min of incubation, DMMB-treated cells showed NBD-PC translocation without significant annexin V binding (Figure 5A, upper right). After prolonged incubation, the cells with the highest NBD-PC translocation also became positive for annexin V (Figure 5A, lower left and right), whereas cells with only moderate NBD-PC translocation remained negative.

To investigate whether the apparent heterogeneity in scrambling activity (and thus in PS exposure) after PDT was due to heterogeneity within the human erythrocytes or due to the experimental conditions employed, we also performed the double label experiments with cells treated with DMMB and red light at a 10-fold lower hematocrit, resulting in a higher light penetration. Under the latter condition, induction of scrambling occurred much more homogeneously (Figure 5B), probably because exposure of the cells occurred in a more homogeneous way. Under these conditions, annexin V binding also occurred only after prolonged incubation, but many more cells became positive (Figure 5B, lower right). Although the sensitivity of detection may be different for the two processes, these results support the notion that increased phospholipid scrambling after PDT of erythrocytes is a prerequisite for PS exposure.

To investigate the role of intracellular free Ca^{2+} in the induction of phospholipid scrambling induced by PDT, erythrocytes were loaded with BAPTA. The intracellular BAPTA

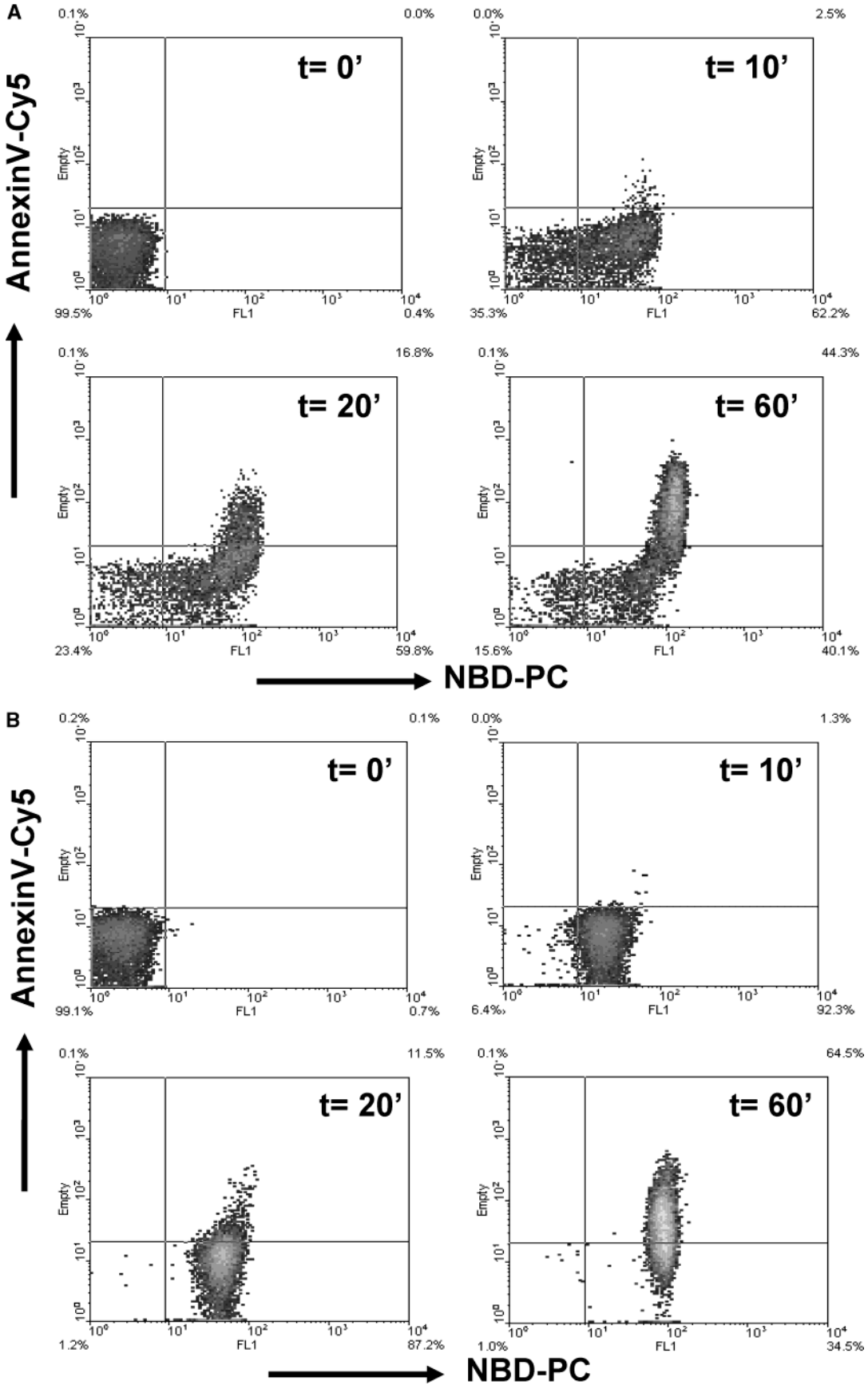

FIGURE 5: Incubation of photodynamically treated erythrocytes with NBD-PC and annexin V−Cy5. Erythrocytes were illuminated in the presence of 20 μM DMMB under two different conditions: (A) standard illumination conditions (30% Ht, 360 kJ/m^2) and (B) after dilution to 3% Ht. In the latter case, the light dose was adjusted to 75 kJ/m^2 to compensate for the higher light transmission at lower hematocrit. After illumination, all cells were diluted to an hematocrit of 0.3% and incubated in the presence of 2.5 mM $CaCl_2$ with NBD-PC and annexin V−Cy5 as described in Materials and Methods. At different time intervals, translocation of NBD-PC and annexin V binding was measured. Results shown are representative for three experiments.

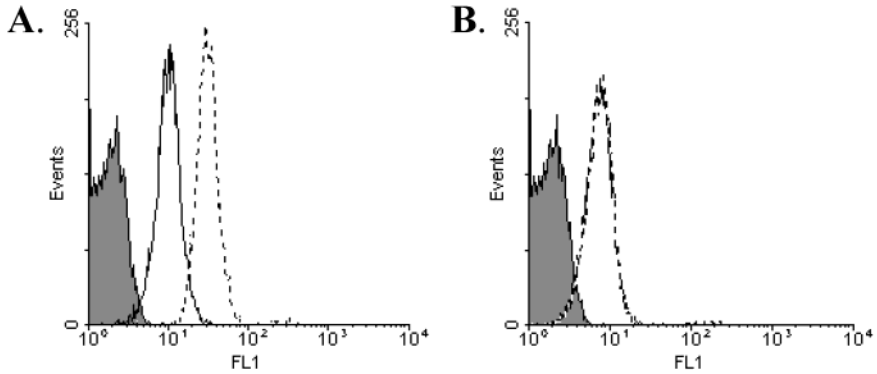

FIGURE 6: Effect of BAPTA loading on the rise in intracellular free Ca^{2+} in human erythrocytes. Erythrocytes (Ht 0.3%) were loaded with 1 μM Fluo-3/AM in the absence or presence of 10 μM BAPTA/AM as described in Materials and Methods. After being washed, cells were incubated with 1 mM $CaCl_2$ and analyzed by flow cytometry, before (closed line) and 3 min after (dashed line) stimulation with 5 μM A23187. Panels: (A) control cells; (B) BAPTA-loaded cells. The gray histograms represent unstimulated cells not loaded with Fluo-3.

concentration was sufficient to prevent a rise in intracellular free Ca^{2+} induced by Ca^{2+} ionophore in the presence of extracellular Ca^{2+} (Figure 6). BAPTA-loaded cells were then subjected to PDT (in the presence of 2.5 mM EGTA). Induction of scrambling (as measured by NBD-PC translocation) was only marginally affected: uptake of the probe after 20 min was 56 ± 3% in control cells and 52 ± 3% in Ca^{2+}-depleted cells (mean ± SEM of four experiments, $P < 0.05$ with the paired *t*-test). Inhibition of PS exposure induced by PDT was somewhat higher (18 ± 4% vs 13 ± 4%, mean ± SEM of four experiments, $P < 0.01$ with the paired *t*-test), but clearly PS exposure still occurred. Conversely, addition of 1 mM $CaCl_2$ to the incubation medium during photodynamic treatment did not enhance the induction of NBD-PC transport activity (data not shown).

Previously, we have shown that red cell damage induced by PDT with DMMB as photosensitizer can be minimized by inclusion of the singlet oxygen scavenger dipyridamole (*14*, *19*). To investigate the involvement of singlet oxygen in the PS exposure and scrambling activity induced by PDT, cells were preincubated with various scavengers of singlet oxygen: azide, trolox (*20*), and dipyridamole (*19*). Trolox and dipyridamole inhibited both responses for more than 75%, while azide appeared to be somewhat less effective (Table 2). These results indicate that generation of singlet oxygen plays an important role in the activation of phospholipid scrambling under the experimental conditions employed in this study.

Table 2: Inhibition of PS Exposure and NBD-PC Uptake by Singlet Oxygen Scavengers

addition	PS-positive cells (%)	NBD-PC uptake (%)
none	10.9 ± 1.5	38.1 ± 3.8
+azide	4.7 ± 1.9	11.6 ± 2.2
+trolox	2.8 ± 1.1	6.6 ± 1.2
+dipyridamole	0.8 ± 0.3	8.1 ± 1.2

[a] Erythrocytes (Ht 30%) were incubated for 10 min at room temperature with azide (10 mM), trolox (4 mM), or dipyridamole (200 μM) prior to addition of DMMB (20 μM) and illumination (360 kJ/m^2). After photodynamic treatment, the extent of PS exposure was determined by annexin V labeling as described under Materials and Methods (mean ± SEM of four experiments). In another set of experiments, cells were diluted after illumination to 5% Ht and incubated with 2 μM NBD-PC (and the indicated concentration of inhibitor) at 37 °C. After 20 min, samples were analyzed for the amount of label translocated to the inner leaflet as described under Materials and Methods. Values were corrected for the amount of fluorescence determined in samples taken at $t = 0$ (amounting to 2.7 ± 1.2%, mean ± SEM of four experiments). All treatments resulted in a significant difference as compared to the control ($P < 0.005$, Student's *t*-test for paired observations).

DISCUSSION

The results of this study show for the first time that phospholipid scrambling can be induced in human erythrocytes by singlet oxygen that is generated upon illumination of the photosensitizer DMMB. This activation leads to exposure of PS on the outer leaflet of the erythrocyte membrane, which might compromise the survival of erythrocytes after transfusion (*8*).

As outlined in the introduction, enhanced PS exposure can be the result of a decreased ability to shuttle PS back to the inner leaflet of the plasma membrane. Although the aminophospholipid translocase is ATP-dependent and ATP levels were affected under our conditions of photodynamic treatment (decreasing to 80 ± 5%, mean ± SEM of four experiments), it is not likely that this decrease is of significance. A similar decrease of ATP induced by a 4 h preincubation at 37 °C in the absence of glucose did not result in inhibition of NBD-PS uptake (data not shown). It cannot be excluded that inhibition of translocase activity by some other mechanism does play a role in the PS exposure observed, but the enhancing effect of NEM pretreatment on PS exposure (Table 1) and the apparent absence of PS exposure in part of the cells (Figure 5) indicate that the aminophospholipid translocase was at least partially active and able to reverse some of the outward movement of PS.

It is not likely that MRP1 contributed to enhanced PS exposure, since indomethacin did not affect annexin V binding after PDT (Table 1). However, our measurements of NBD-PC translocation and NBD-PS translocation (in the presence of NEM) clearly demonstrated that phospholipid scrambling is induced after PDT. As can be seen from the data depicted in Figure 5, the balance between outward and inward movement of PS is further shifted to outward movement by incubation at 37 °C. Indeed, scrambling activity as measured by NBD-PC translocation was 3-fold higher at 37 °C than at room temperature, whereas aminophospholipid translocase activity (measured in untreated erythrocytes) showed a much lower increase at this elevated temperature (data not shown).

The observation that phospholipid scrambling could be prevented by the presence of various singlet oxygen scavengers (Table 2) indicates that singlet oxygen is important for this activation to occur. It is of interest to note that treatment with hydrogen peroxide does not result in scrambling activity but, instead, inhibits this activity (Table 1 and ref *12*). Although we obtained clear evidence supporting induction of scrambling as the underlying mechanism of enhanced PS exposure, it has been shown that enhanced lipid peroxidation also induces binding of annexin V (*21*). The experimental conditions used in our study did result to a minor degree of lipid peroxidation [as measured with BODIPY−C_{11} as probe (*22*)], but a similar degree of lipid peroxidation induced by moderate treatment with *tert*-butyl hydroperoxide (*t*-BHP) did not result in significant annexin V binding nor in significant uptake of NBD-PC (data not shown).

The best known activators of phospholipid scrambling in erythrocytes thus far have been Ca^{2+} and acidification (*9*, *11*, *23*). The effect of photodynamic treatment was not due to acidification of the erythrocytes, since the intracellular pH was not affected (data not shown). It also appeared not to be caused by changes in intracellular Ca^{2+}, since the induction of PS exposure and NBD-PC translocation were only marginally influenced by Ca^{2+} depletion. In nucleated cells, enhanced PS exposure is one of the hallmarks of apoptosis, and in lymphocytes, it has been shown that phospholipid scrambling occurs upon induction of apoptosis, which also seems to be independent of Ca^{2+} (*16*).

Recently, the cDNAs of four homologous proteins have been cloned that have been proposed to be catalysts of phospholipid scrambling (*11*, *24*, *25*). However, absence of the first identified member of this protein family (PLSCR-1) in murine erythrocytes does not prohibit phospholipid scrambling induced by Ca^{2+} (*26*), while it does affect signal transduction via EGF receptors (*27*). It therefore remains to be determined what proteins are involved in phospholipid scrambling in human erythrocytes and which of these are activated by PDT.

ACKNOWLEDGMENT

The authors greatly appreciate the help of Mr. R. Vlaar in preparing the red blood cell suspensions.

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BI035637K