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Original Contribution

Membrane cholesterol in the regulation of aminophospholipid asymmetry and phagocytosis in oxidized erythrocytes

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Abstract

Cholesterol is known to affect several membrane functions, including membrane susceptibility to oxidative stress. In order to gain a better understanding of the relationship between cholesterol contents, structural integrity, and degree of survival in oxidatively stressed erythrocytes, here we analyzed the transbilayer phospholipid distribution, the morphology, and the degree of clearance observed in cholesterol-modified (enriched or depleted) and unmodified (control) erythrocytes exposed to *tert*-butylhydroperoxide. We report that the modification of cholesterol contents in erythrocytes promotes the externalization of phosphatidylserine (PS) to the membrane surface, which is consistent with a concomitant inhibition of aminophospholipid translocase (APLT) and an increased uptake of modified erythrocytes by macrophages. Moreover, cholesterol depletion modifies the transbilayer aminophospholipid distribution induced by oxidative stress to a great extent, significantly increasing PS externalization, which is associated with the strongest decrease in APLT activity. The loss of normal PS asymmetry is positively correlated with enhanced phagocytosis, and an increase in echinocyte forms is observed in all oxidized erythrocytes. We envisage that PS externalization could be due, at least in part, to the decrease in APLT activity induced by oxidative stress, the activity of which is also dependent on membrane cholesterol contents.

Keywords: Aminophospholipid asymmetry; Aminophospholipid translocase; Cholesterol; Erythrocytes; Erythrophagocytosis; Oxidative damage; *tert*-Butyl hydroperoxide; Free radicals

Normal circulating blood cells exhibit an asymmetric distribution of phospholipids in the membrane, in which phosphatidylserine (PS) and phosphatidylethanolamine (PE) are mainly located in the inner leaflet, whereas phosphatidylcholine (PC) and sphingomyelin are enriched in the outer leaflet [1,2]. Whereas this asymmetry is the rule for normal blood cells, a loss of PS asymmetry has been reported to occur in blood cells associated with several pathologic conditions, such as diabetes [3], hemolytic anemias such as sickle cell diseases [4], and some cases of β-thalassemia [5], as well as with oxidative processes induced in aged [6] or oxidatively stressed [7] erythrocytes.

Membrane lipid asymmetry is dependent on ATP and is dissipated by sulfhydryl-oxidizing reagents, an elevation in intracellular Ca^{2+}, and proteolysis, each of which may be affected by oxidative stress [4,8]. The Mg^{2+}-ATP-dependent aminophospholipid translocase (APLT) helps to maintain membrane asymmetry by moving PS, and to a lesser extent PE, from the outer to the inner leaflet of the plasma membrane against the concentration gradient, thus compensating for any escape of PS or PE to the outer leaflet [9]. Phospholipid scramblase is inactive in resting cells but may become active

Abbreviations: Ac-DEVD-CHO, caspase 3 inhibitor; AU, absorbance unit; APLT, aminophospholipid translocase; BCD, β-cyclodextrin; Ch, cholesterol; DCF, 2′,7′-dichlorofluorescein; FITC, fluorescein isothiocyanate; Hb, hemoglobin; MBCD, methyl-β-cyclodextrin; MDA, malonyldialdehyde; NBD-PS, 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycerol-3-[phospho-L-serine]; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; PVP, polyvinylpyrrolidone; RBCs, red blood cells (erythrocytes); ROS, reactive oxygen species; *t*-BuOOH, *tert*-butylhydroperoxide; TBARS, thiobarbituric acid-reactive substances.

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under particular conditions of cellular activation or in response to apoptotic stimuli. Activation of phospholipid scramblase results in an enhanced bidirectional transmembrane migration of all membrane phospholipids [10]. The activation of scramblase, accompanied by simultaneous inhibition of APLT, causes a collapse of transmembrane lipid asymmetry. The most prominent feature of this collapse is the exposure of PS in the external leaflet of the plasma membrane. This phenomenon is of major importance for normal hemostasis because surface-exposed PS is required to promote both the assembly and the catalytic efficiency of several lipid-dependent coagulation reactions [11,12]. In addition, PS externalization, which also occurs in apoptotic [12,13] and tumorigenic [12,14] cells, is preceded by its selective oxidation, serving as a signal for the recognition and clearance of aberrant cells by the reticuloendothelial system [12,15–17]. Furthermore, the dynamic process of phospholipid translocation may play an important role in cellular events such as the shedding of microvesicles [18] and fusion processes [19]. The asymmetric distribution of aminophospholipids also has significant effects on cell shape and mechanical membrane stability through the interactions of skeletal proteins with the inner leaflet of the lipid bilayer [20,21].

Oxidative stress and the inactivation of the systems that maintain phospholipid asymmetry play a central role in the events leading to PS exposure and death and removal of affected cells [6,7,16,22]. In this sense, APLT is redox-dependent and sensitive to the oxidation and alkylation of its SH-groups [23,24]. This apparent sensitivity to sulfhydryl modification, which could be a result of oxidative stress together with a possible decrease in ATP, would predict impaired APLT activity in oxidatively challenged red cells. Significant decreases in APLT activity have been reported during in vivo and artificial aging of human erythrocytes [6] or in red blood cells exposed to *tert*-butylhydroperoxide (*t*-BuOOH) [7,25].

The perturbation of membrane cholesterol contents and structural organization has been implicated in the pathogenesis of a number of human diseases, many of which are also linked to conditions of increased oxidative stress, such as cardiovascular and neurodegenerative disorders [26]. In hypercholesterolemic conditions, the cholesterol contents of blood cells have been shown to increase, and this increase has been reported to cause enhanced susceptibility to peroxidation [27]. The susceptibility of cells to oxidative stress is dependent on the nature and physical state of the membrane lipid bilayer. Cholesterol directly modulates the physical properties of lipid bilayers, altering membrane responses to degenerative process, including lipid peroxidation [28,29]. We have recently reported that the modification of cholesterol levels in rabbit erythrocytes, at concentrations above or below physiological contents, has stimulating effects on reactive oxygen species (ROS)-induced peroxidation, also increasing oxidative damage in peroxidized erythrocytes [30].

Taking into account the increased susceptibility to oxidative damage observed in cholesterol-modified erythrocytes [30] and the correlation between oxidative events and the loss of phospholipid asymmetry [6,7,16,22], the goal of the present study was to investigate the influence of cholesterol levels in the membrane lipid asymmetry of erythrocytes exposed to oxidative stress. Here we demonstrate for the first time that the oxidative damage to cholesterol-modified erythrocytes produces a loss of phospholipid asymmetry, with increased externalization of PS and enhanced erythrophagocytosis, all accompanied by a decrease in APLT activity.

Materials and methods

Materials

Annexin V–fluorescein isothiocyanate conjugate (FITC–annexin V), t-BuOOH, cholesterol (Ch), β-cyclodextrin (BCD), dichlorodihydrofluorescein diacetate (DCFDA), 3,3′-dimethoxybenzidine, hemoglobin (Hb), methyl-β-cyclodextrin (MBCD), N-acetyl-Asp-Glu-Val-Asp-al (Ac-DEVD-CHO, caspase 3 inhibitor), phenylmethanesulfonyl fluoride (PMSF), phospholipase A_2 from bee venom, and polyvinylpyrrolidone (PVP) were supplied by Sigma Chemical Co. (Sigma–Aldrich Química, Madrid, Spain). Glutaraldehyde solution 25% and sodium dithionite were obtained from Fluka (Sigma–Aldrich Química). Phospholipid standards and Phospray were from Supelco, Inc. (Sigma–Aldrich Química). 1-Palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycerol-3-[phospho-L-serine] sodium salt (NBD–phosphatidylserine, NBD-PS) was supplied by Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Malondialdehyde-bis(diethylacetal) and thin-layer chromatography plates (Silicagel G, type 60) were obtained from Merck (Darmstadt, Germany). RPMI 1640 medium, L-glutamine, penicillin, streptomycin, and fetal bovine serum were purchased from BioWhittaker (Innogenetics, Barcelona, Spain). Giemsa and the remaining products were from Panreac (Barcelona, Spain). All organic solvents were of high quality and twice-distilled.

Preparation of erythrocytes

Blood was obtained from the ear of adult male rabbits by venipuncture and collected in citrate/phosphate/dextrose/adenine at a ratio of 7:1 (v/v). After centrifugation (200*g*, 15 min), erythrocytes were isolated; washed twice in 3 volumes of a solution composed of 150 mM NaCl, 5 mM sodium phosphate buffer, pH 7.5 (PBS), at 700*g* for 6 min; and packed at 2500*g* for 5 min.

The total Hb content in erythrocyte suspensions was measured as cyanmethemoglobin, as indicated previously [30].

Modification of cholesterol levels in erythrocytes

Cholesterol enrichment was performed by incubation of the erythrocytes (10% hematocrit) in PBS solution containing 3.5% (w/v) PVP and 0.5 mg cholesterol at room temperature with gentle shaking for 4 h. The mixture was then centrifuged at 1000*g* for 5 min, washed once with PBS, and packed at 2500*g* for 5 min [30,31].

Cholesterol depletion was performed by treatment of the erythrocytes (13% hematocrit) with 5 mM MBCD at 37°C with

gentle shaking for 1 h, after which the cells were washed under the conditions described above [30].

The extent of cell lysis after cholesterol modification was estimated by measuring the release of hemoglobin from the treated cells at 418 nm absorbance in the 1000*g* supernatant, comparing the data thus obtained with those from a completely lysed preparation.

Total lipids were extracted from packed unmodified and cholesterol-modified erythrocytes before and after *t*-BuOOH oxidation with mixtures of isopropanol/chloroform, as previously reported [32]. Cholesterol (Ch), phospholipids (PL), and the molar ratio of cholesterol to total phospholipids (Ch/PL) were determined in the lipid extracts, as indicated elsewhere [30].

In vitro oxidation of erythrocytes

Erythrocyte (13% hematocrit in 1.5 ml of mixture) oxidation was carried out in PBS with different concentrations of *t*-BuOOH at 37°C for 30 min with gentle shaking. After treatment, oxidized cells were obtained by centrifugation at 2500*g* for 5min, washed twice in PBS, and then used for other purposes [30,33].

Determination of intracellular ROS and thiobarbituric acid-reactive substances (TBARS)

Nonoxidized erythrocytes (10% hematocrit) were incubated in PBS in the presence of 10 μM DCFDA, a nonfluorescent compound that freely permeates cells, at 37°C for 30 min [30,33]. Then, erythrocytes were centrifuged and washed twice in PBS at 1000*g* for 5 min. The determination of ROS production by exposure to *t*-BuOOH was performed in 2.7 ml of a mixture containing PBS, erythrocytes loaded with DCFDA (0.25% hematocrit), and 0.2 mM *t*-BuOOH, placed in a quartz cuvette in a Hitachi F-4010 fluorimeter equipped with a magnetic stirrer and thermostated at 37°C. Under these conditions, DCFDA is hydrolyzed to 2′,7′-dichlorodihydrofluorescein, which is suitable for oxidation by ROS to produce fluorescent 2′,7′-dichlorofluorescein (DCF). This fluorescence was monitored at 530 nm after excitation at 495 nm. In the absence of the oxidant, no increases in DCF fluorescence were observed. Data points were taken every 30 s over 15 min and exported to Excel spreadsheet software for analysis.

The lipid peroxidation levels of oxidized erythrocytes were evaluated by TBARS determination. Aliquots (1 ml) of oxidation medium were added to mixtures of trichloroacetic acid–sodium arsenite, as described previously [30,33]. TBARS concentrations were measured in a spectrophotometer at 535 nm and data were expressed as malonyldialdehyde (MDA) equivalents, using malondialdehyde-bis(diethylacetal) (0.2–7 nmol) as standard.

Phospholipase treatment and phospholipid analysis

Membrane phospholipid asymmetry in erythrocytes was determined by phospholipid digestion of the outer monolayer following a modification of the procedure of Dumaswala et al. [34]. Briefly, 0.5 ml of packed erythrocytes was suspended in 2 ml of Hepes-buffered saline 1 (HBS_1; 10 mM Hepes, 150 mM KCl, pH 7.4) containing 13 mM $CaCl_2$ and 0.3 mM $MgCl_2$. Phospholipase A_2 from bee venom, 0.1 ml of a 1000 IU/ml solution, was added to each sample, and the suspension was incubated for 20 min at 37°C. Erythrocytes were collected by centrifugation, and the degree of hemolysis was estimated in the supernatant by spectrophotometric reading at 418 nm. Packed erythrocytes were suspended in 0.25 ml of HBS_1 buffer containing 10 mM EDTA and kept on ice. This procedure of phospholipase digestion has been shown to produce less than 6% hemolysis in nonoxidized erythrocytes, in control and cholesterol-enriched erythrocytes oxidized with 0.5 mM *t*-BuOOH, and in cholesterol-depleted erythrocytes exposed to 0.25 mM *t*-BuOOH. The procedure gives hydrolysis of 90% in PC and PE and of 48% in PS in solubilized lipids (data of three separate assays), taking into account the above data to estimate the real phospholipid distribution in the membrane outer monolayer.

Total lipids from erythrocytes treated with phospholipase A_2 were extracted with mixtures of isopropanol/chloroform [32]. Phospholipid classes from lipid extracts were separated by two-dimensional thin-layer chromatography and quantified by phosphorus analysis of the lipid spots scraped from each plate as described before [30].

Annexin V labeling of erythrocytes and flow-cytometric analyses

Erythrocyte labeling and data acquisition were performed essentially as described earlier [25,35]. Briefly, erythrocytes were suspended at 0.04% hematocrit in Hepes-buffered saline 2 (HBS_2; 10 mM Hepes, 145 mM NaCl, pH 7.4) containing 2.5 mM $CaCl_2$ and labeled with 60 ng/ml FITC–annexin V (final concentration). After an incubation in the dark for 30 min at room temperature, the cells were pelleted at 10,000*g* for 10 s, and the supernatant was removed. The cells were resuspended at 0.04% hematocrit in HBS_2 containing 2.5 mM $CaCl_2$.

Information about 3×10^5 events/sample aliquot was collected using a FACSCalibur flow cytometer and the CellQUEST software program (Becton–Dickinson (BDB), San Jose, CA, USA). For data analysis, the Paint-A-Gate Pro and CellQUEST software programs (BDB) were used.

Forward (FSC) and sideward (SSC) scatter profiles were used to define the region of the intact red cell population. Only those events that were within the intact erythrocyte region (>92%) were analyzed for fluorescence intensity. The percentage of annexin V-positive erythrocytes was determined from the fluorescence signal in excess of that obtained with respect to a negative (unlabeled) control sample aliquot, which was run for each sample to consider the autofluorescence properties of the cells after the incubation period.

Phagocytosis of erythrocytes by J774 macrophages

Phagocytosis assays were carried out according to the procedure of Pradhan et al. [36]. J774 mouse macrophages (Animal Cell Culture Service of the Centro de Investigaciones

Biológicas del CSIC, Madrid, Spain) were added to 24-well (18-mm) plates (3×10^5 cells per well) and grown in RPMI 1640 medium containing 10% fetal bovine serum, 4 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) followed by incubation at 37°C in 5% CO_2 24 h before the assay. Erythrocytes (15×10^6 cells) suspended in 450 μl of serum-free culture medium were added to 90% confluent macrophage monolayers previously rinsed with serum-free culture medium and then incubated at 37°C for 1 h in 5% CO_2. After this, the supernatants were aspirated and the wells were washed twice with ice-cold PBS and incubated for 5 min at room temperature with 140 mM NH_4Cl, 17 mM Tris–HCl, pH 7.2, to lyse unphagocytosed erythrocytes. After treatment of the wells with cold methanol, ingested erythrocytes (previously stained with 0.0125% 3,3′-dimethoxybenzidine containing 0.006% H_2O_2 for 10 min, rinsed once with PBS, and counterstained with Giemsa stain) were examined with light microscopy. Three hundred macrophages were counted per well. Two to four replicate wells were evaluated in each experiment. Macrophages that had phagocytosed one or more erythrocytes were considered to be positive macrophages. The phagocytic index was expressed as the percentage of positive macrophages.

Measurement of aminophospholipid translocase activity

APLT activity was measured using modifications of the procedure of Tyurina et al. [16]. Erythrocytes, oxidized or nonoxidized, were suspended in incubation buffer (90 mM KCl, 45 mM NaCl, 44 mM sucrose, 20 mM glucose, and 10 mM Tris–HCl, pH 7.4) containing 500 μM PMSF at 0.8% hematocrit, placed in ice water for 10 min, labeled with 10 μM NBD-PS (final concentration), and incubated for 10 min at 4°C. Labeled cells were centrifuged and resuspended again at the same density in the incubation buffer containing PMSF. Cell suspensions were placed in a water bath at 28°C to initiate the internalization of labeled PS, and 300-μl aliquots of erythrocyte suspension were removed at 10 min and placed in 2.2 ml of incubation buffer containing PMSF. Fluorescence (ex 470 nm, em 540 nm) was then recorded (within 180 s) before and after the addition of 40 μl of fresh 0.5 M sodium dithionite solution at 10 s. Samples without dithionite were measured to obtain total fluorescence intensity (FL_{total}). Samples not incubated at 28°C were also used to obtain the basal fluorescence intensity (FL_0). Internalized fluorescence (internalized NBD-PS) determined at various times (FL_t) was normalized as a percentage of total fluorescence with the equation % internalized = $(FL_t - FL_0)/(FL_{total} - FL_0) \times 100$, and APLT activity was expressed as a percentage of NBD-PS internalized. In other cases, APLT activity was expressed as pmol NBD-PS internalized/min·mg Hb, using a calibration curve made using several NBD-PS concentrations (2–40 pmol) in a mixture containing hemolyzed erythrocytes, measuring their fluorescence without sodium dithionite.

Hemolysis and isotonic stability measurements

The percentage of hemolysis in oxidized erythrocytes was estimated by measuring the light scattering produced in the erythrocyte suspension at 700 nm [37]. Isotonic stability was determined by mixing 50 μl of packed erythrocytes with 1.25 ml of 154 mM NaCl. After 16 h at room temperature, the samples were centrifuged at 3500*g* for 5 min and isotonic stability was expressed as percentage of hemolysis in the supernatant of each sample, measured as cyanmethemoglobin absorbance at 540 nm [30]. Cholesterol-depleted erythrocytes (Ch/PL molar ratio of 0.77 ± 0.07) used in the isotonic stability studies were obtained by treatment with 5 mM BCD, performed under conditions identical to those described above for cholesterol depletion (see "Modification of cholesterol levels in erythrocytes" in this section).

Morphology

For morphological studies, packed erythrocytes were diluted 50 times in 1% glutaraldehyde. After fixation for at least 15 min at 4°C, erythrocytes were washed once with PBS and then examined by light microscopy. Morphological changes were considered to have occurred in erythrocytes that contained more than one score on their surface. The average of altered erythrocytes in a field of 100 cells was defined as the morphological index (percentage of altered cells).

Statistical analyses

Data are expressed as means ± SD. Analysis of variance, using the SPSS program for MS Windows (version 13.0.1), was performed for statistical analyses. Differences were considered statistically significant when $p < 0.05$.

Results

Rabbit erythrocytes containing different membrane cholesterol levels were used to study the effects of the oxidative stress induced by *t*-BuOOH on asymmetric phospholipid distribution and their repercussions on cell viability. Cholesterol enrichment afforded increases in cholesterol contents of 89% [6.27 ± 1.24 nmol/mg Hb (Ch/PL molar ratio of 1.41 ± 0.12) vs 3.31 ± 0.38 nmol/mg Hb in control erythrocytes (Ch/PL molar ratio of 0.84 ± 0.10)]. The cholesterol depletion elicited by incubation of the erythrocytes with 5 mM MBCD yielded losses of about 65% of the cholesterol contents (1.15 ± 0.14 nmol/mg Hb with a Ch/PL molar ratio of 0.43 ± 0.09). These treatments produced minimal hemolysis, always less than 2.5%.

The oxidative process induced by *t*-BuOOH significantly increased lipid peroxidation (measured as TBARS formation) and ROS generation in the different erythrocyte types, as reported recently by us [30]. Fig. 1 shows the percentage phospholipid distribution in the membrane outer monolayer in control and cholesterol-modified erythrocytes exposed to *t*-BuOOH. The membrane outer monolayer in rabbit erythrocytes contained 78% PC, 11% PE, and <1% PS, which is in accordance with the values reported in other erythrocyte membranes [1]. The modification of cholesterol contents in rabbit erythrocytes produced changes only in the amino-

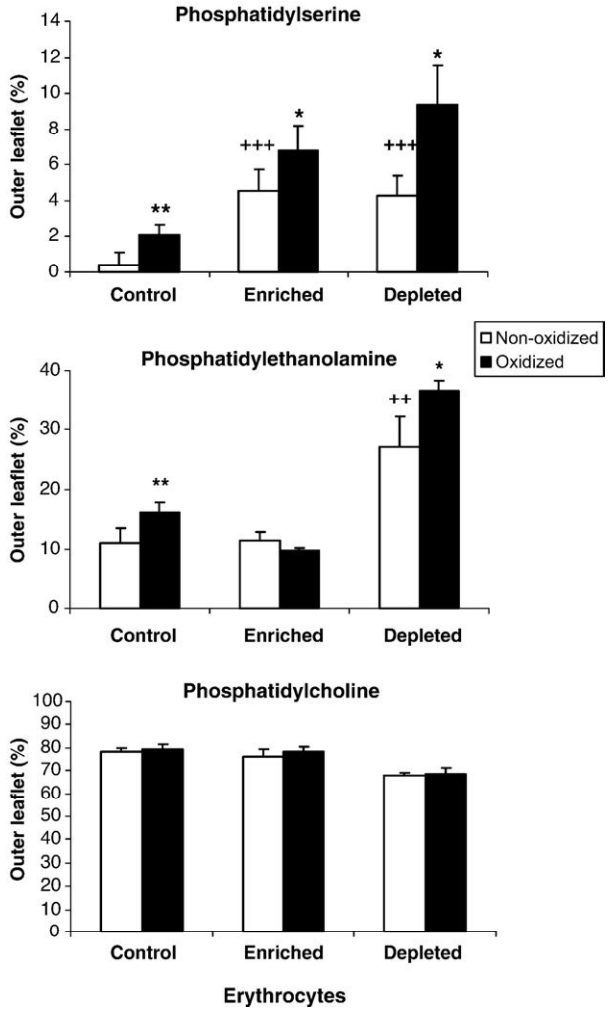

Fig. 1. Phospholipid distribution in the membrane outer monolayer in control (n = 6) and cholesterol-modified (cholesterol-enriched, n = 7; cholesterol-depleted, n = 7) oxidized and nonoxidized erythrocytes. The distribution, expressed as a percentage, was analyzed by phospholipid digestion of the membrane outer monolayer after exposure of the erythrocytes to bee venom phospholipase A_2 for 20 min at 37°C. Oxidations were carried out at 0.5 mM *t*-BuOOH in control and cholesterol-enriched erythrocytes and at 0.25 mM *t*-BuOOH in cholesterol-depleted erythrocytes. Data are means ± SD of n independent experiments. Comparisons are between oxidized and nonoxidized for each erythrocyte group. Differences were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$. In nonoxidized erythrocytes, the differences were significant in each cholesterol-modified erythrocyte group with respect to the control at $^{++}p < 0.01$, $^{+++}p < 0.001$.

phospholipid distribution of their membranes: (i) PS exposure in the outer membrane was increased by about 11-fold in cholesterol-enriched and cholesterol-depleted erythrocytes with respect to the control group and (ii) PE externalization was enhanced by only about 2.5-fold in cholesterol-depleted erythrocytes. However, no significant changes were observed in PC distribution after modification of the cholesterol contents. The effects of oxidative treatment on membrane phospholipid distribution were assessed only at 0.5 mM *t*-BuOOH in control and cholesterol-enriched erythrocytes and at 0.25 mM *t*-BuOOH in cholesterol-depleted cells, because only under these conditions was less than 10% hemolysis detected after phospholipase A_2 treatment. Under the above conditions, the oxidative damage induced by *t*-BuOOH produced significant changes only in aminophospholipid distribution (Fig. 1): (i) PS exposure to the membrane surface increased significantly in oxidized control (5.2-fold), cholesterol-enriched (1.5-fold), and cholesterol-depleted (2.2-fold) erythrocytes compared to their respective nonoxidized group, and (ii) PE externalization was always less pronounced after oxidation (1.5-fold in control and 1.3-fold in cholesterol-depleted groups).

The presence of PS in the outer monolayer of the red blood cell (RBC) membrane was also analyzed by flow cytometry using FITC-labeled annexin V (Fig. 2). Populations of intact RBCs were defined by their physical parameters in nonoxidized control and cholesterol-modified erythrocytes and in oxidized control and cholesterol-modified erythrocytes, i.e., SSC (cell complexity/density) vs FSC (cell volume) (see insets of the different plots in Fig. 2A). Cell debris was characterized by lowest SSC and lowest FSC (data not shown), whereas viable cells showed higher FSC, corresponding to cells with a normal morphology. Cholesterol modification and *t*-BuOOH treatment up to 1 mM concentration in control and cholesterol-enriched erythrocytes and up to 0.5 mM in cholesterol-depleted erythrocytes maintained almost intact RBCs (> 90%). We noted that the depletion of cholesterol in nonoxidized and oxidized RBCs produced cell populations with forward and side scatter distributions different from those observed in the control and cholesterol-enriched groups. Events that correlated with intact RBCs labeled with annexin V–FITC were analyzed for fluorescence intensity (see cytofluorimetric profiles in Fig. 2A). In the absence of the fluorophore, the RBCs did not show any appreciable fluorescence in flow-cytometric analysis (data not shown), but were used as negative controls. The treatment of RBCs with increased *t*-BuOOH concentrations resulted in a progressive binding of annexin V–FITC to the cell (see cytofluorimetric histograms in Fig. 2B). In the absence of the oxidant, minimal binding (< 0.2%) of annexin V–FITC to cholesterol-modified RBCs was observed, which is interpreted in terms of the notion that the exposed PS at the membrane surface is minimally available for binding with annexin V. The externalization of PS (expressed as percentage of erythrocytes labeled with FITC–annexin V) increased progressively in control erythrocytes exposed to 0.5 (6-fold), 0.75 (29-fold), and 1 mM (46-fold) *t*-BuOOH concentrations with respect to nonoxidized cells. Less externalization of PS was observed in oxidized cholesterol-enriched erythrocytes, with increases in annexin V-labeled cells of 6-, 23-, and 30-fold at 0.5, 0.75, and 1 mM *t*-BuOOH concentration, respectively. Cholesterol depletion rendered erythrocytes more sensitive to oxidative stress, because lower oxidant concentrations yielded higher PS externalizations (13-fold at 0.25 mM and 34-fold at 0.5 mM *t*-BuOOH) than in other oxidized RBCs. At the same oxidant concentration, the cholesterol-depleted group showed a higher degree of PS exposure than the control erythrocytes, with increases of 15- and 11-fold at 0.25 and 0.5 mM *t*-BuOOH concentration, respectively.

Fig. 3 shows that the progressive increase in the oxidative stress in RBCs results in an exponential increase in PS

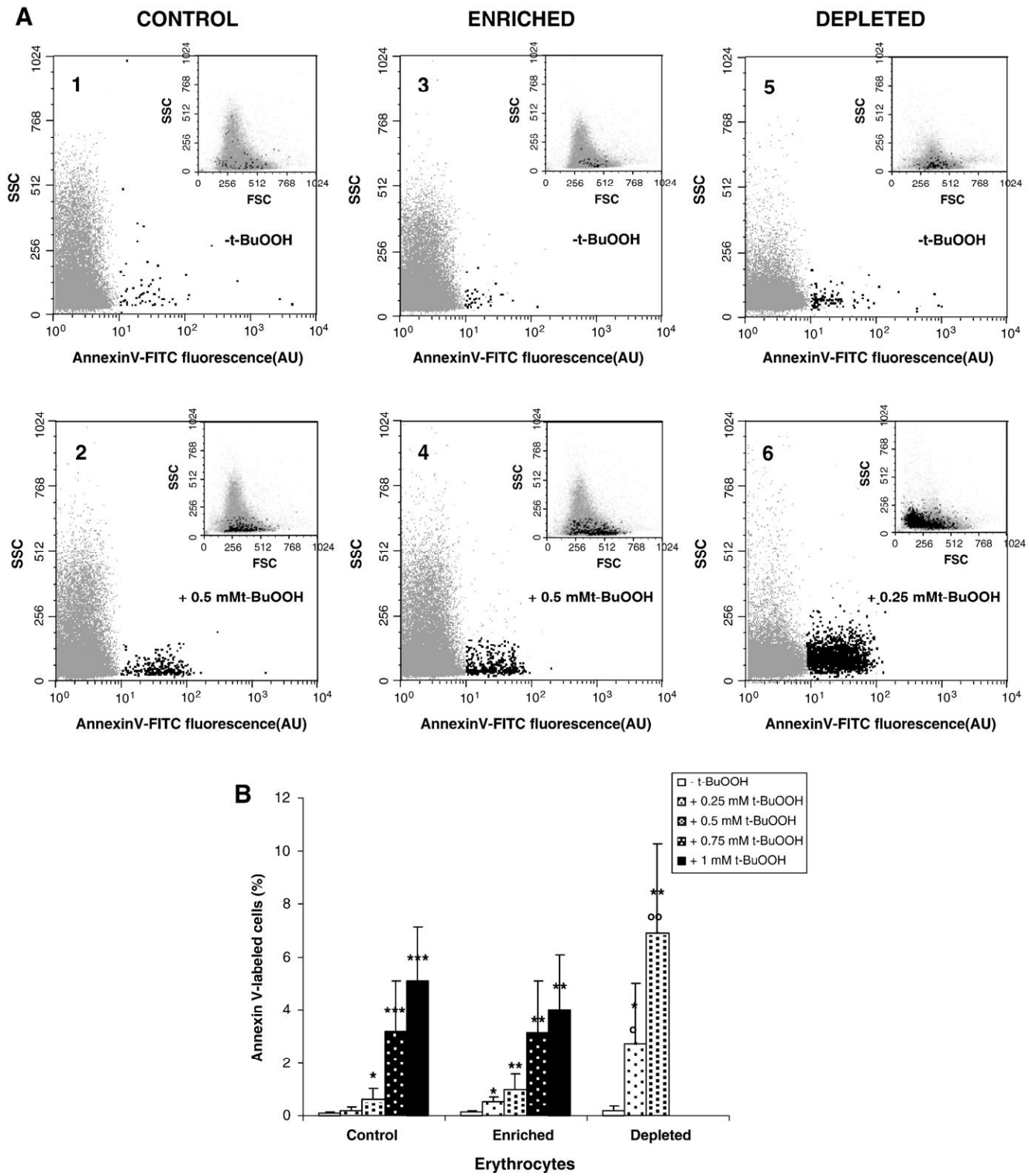

Fig. 2. Flow cytometry analysis of PS externalization in oxidized erythrocytes with different cholesterol contents. (A) Flow-cytometric profiles of different erythrocyte types labeled with annexin V–FITC, as indicated under Materials and methods: control erythrocytes, untreated (1) or treated (2) with 0.5 mM *t*-BuOOH; cholesterol-enriched erythrocytes, untreated (3) or treated (4) with 0.5 mM *t*-BuOOH; and cholesterol-depleted erythrocytes, untreated (5) or treated (6) with 0.25 mM *t*-BuOOH. Erythrocyte populations were identified by their light scatter properties (SSC/FSC; see inset in each dot plot), and only populations with > 92% of intact cells were used for the cytofluorimetric analyses. Each dot plot is representative of three independent experiments. (B) Histograms for quantifying PS externalization by the binding of annexin V–FITC to control, cholesterol-enriched, and cholesterol-depleted erythrocytes exposed to various *t*-BuOOH concentrations. Results are expressed as percentages of annexin V-labeled cells: means ± SD of eight separate assays. Comparisons are between nonoxidized erythrocytes (−*t*-BuOOH) and each oxidized group for the same erythrocyte type; the differences were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. For the same oxidant concentration, the differences were significant in each cholesterol-modified erythrocyte group with respect to the control at $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$.

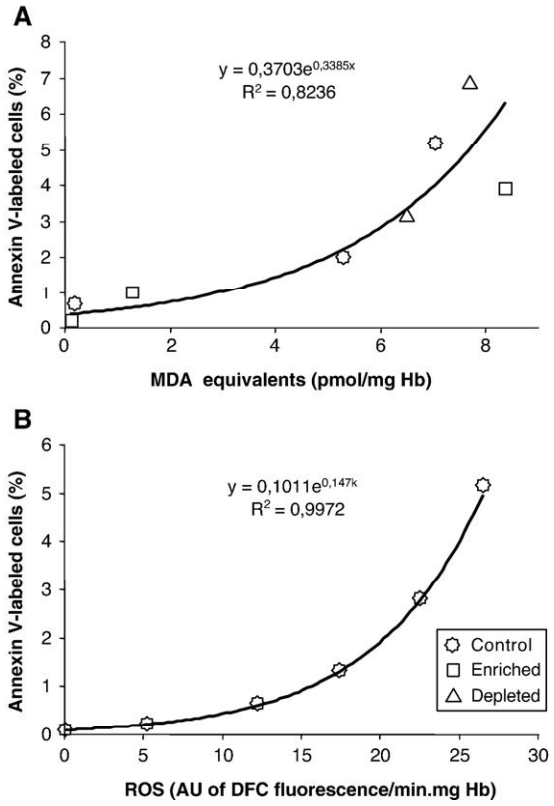

Fig. 3. Relationship between PS externalization (expressed as percentage of cells labeled with annexin V–FITC) and oxidative stress, expressed as (A) MDA equivalents or (B) ROS generated in erythrocytes. Each plot point is the mean of three separate assays performed in control, cholesterol-enriched, or cholesterol-depleted erythrocytes treated with progressive (0–1 mM) *t*-BuOOH concentrations (A) and in control erythrocytes exposed to 0–1 mM *t*-BuOOH (B).

externalization, with about 5% of annexin V-labeled cells in the presence of 8 pmol MDA equivalents (Fig. 3A) or with 26 AU of DFC fluorescence (ROS, Fig. 3B) per milligram of hemoglobin.

Because phagocytosis relies on the recognition of externalized PS on the surface of abnormal cells, we also studied the effects of *t*-BuOOH on the phagocytosis of control and cholesterol-modified erythrocytes by J774 macrophages (Fig. 4A). Relatively low levels of phagocytosis-positive cells (expressed as positive macrophages, < 2%) were detected when nonoxidized control erythrocytes were incubated with macrophages. Cholesterol-modified cells were phagocytosed at a higher (~ 10-fold in both cholesterol-modified groups) level than control cells. Remarkably, control erythrocytes treated with *t*-BuOOH were readily ingested by macrophages, yielding significant increases of 4-fold at 0.5 mM, 8-fold at 1 mM, and 14-fold at 2 mM in the number of phagocytosis-positive cells. The oxidative treatment in cholesterol-modified cells also increased the degree of phagocytosis, in both the cholesterol-enriched (1.3-fold at 0.5 mM *t*-BuOOH, 2.0-fold at 1 and 2 mM *t*-BuOOH) and the cholesterol-depleted (1.6-fold at 0.25 mM *t*-BuOOH) groups. Fig. 4B shows that the presence of 30 μM Ac-DEVD-CHO, a well-known inhibitor of caspase 3 activity, in the medium without oxidant produced a partial inhibition (~64% in cholesterol-enriched and 77% in cholesterol-depleted erythrocytes) of the number of phagocytosis-positive cells. The phagocytosis induced by *t*-BuOOH in the different erythrocyte types was also blocked by caspase 3 inhibitor,

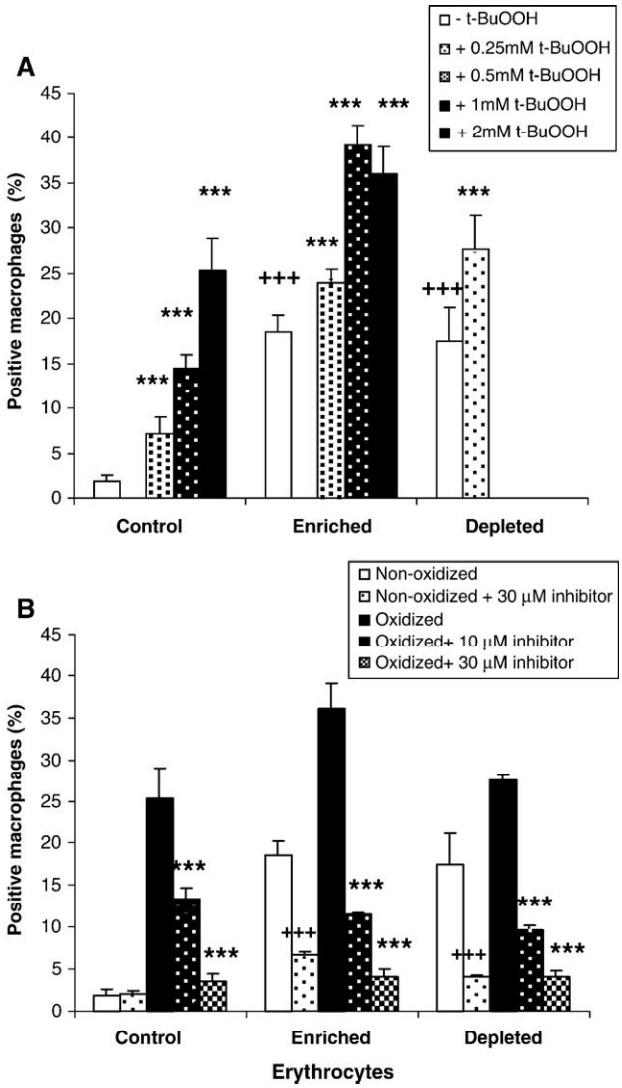

Fig. 4. Cholesterol contents in erythrocytes modify the degree of erythrocyte phagocytosis induced by oxidative stress. The phagocytic index is expressed as a percentage of positive macrophages, as described under Materials and methods. (A) Phagocytosis by J774 mouse macrophages of control (n = 8), cholesterol-enriched (n = 8), and cholesterol-depleted (n = 11) erythrocytes oxidized with different *t*-BuOOH concentrations. Data are means ± SD of n separate experiments. See legend of Fig. 1 for statistical comparisons, including significance at ***$p < 0.001$, +++$p < 0.001$. (B) Effect of caspase 3 inhibitor on the phagocytosis of control and cholesterol-modified erythrocytes exposed to *t*-BuOOH. Erythrocytes were incubated alone or with caspase 3 inhibitor (Ac-DEVD-CHO) for 1 h 30 min at room temperature, subsequently oxidized (control and cholesterol-enriched erythrocytes with 2 mM *t*-BuOOH and cholesterol-depleted erythrocytes with 0.25 mM *t*-BuOOH) or not oxidized, and finally incubated with macrophages as indicated previously. Results are means ± SD of three separate assays. In each erythrocyte group: (i) for comparisons between nonoxidized erythrocytes without inhibitor vs nonoxidized erythrocytes with inhibitor, differences were considered significant at +++$p < 0.001$; (ii) for comparisons between oxidized erythrocytes without inhibitor vs oxidized erythrocytes with inhibitor, differences were considered significant at ***$p < 0.001$.

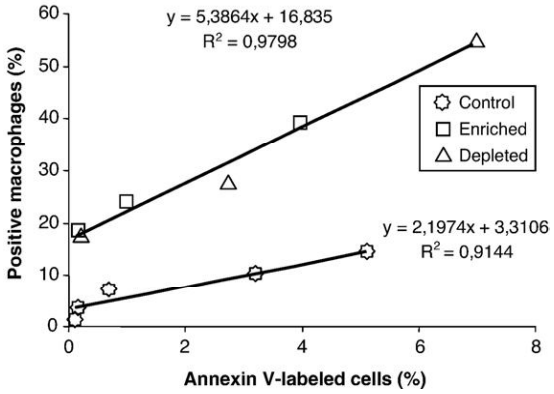

Fig. 5. Correlations between PS externalization (expressed as a percentage of cells labeled with annexin V–FITC) and erythrophagocytosis (percentage of positive macrophages as described under Materials and methods) observed in control and cholesterol-modified erythrocytes. Each point is the mean of three separate determinations performed in control, cholesterol-enriched, or cholesterol-depleted erythrocytes treated with progressive (0–1 mM) *t*-BuOOH concentrations.

almost total inhibition being achieved at 30 μM concentration in all cases. Lower inhibitor concentrations (10 μM) decreased phagocytosis more efficiently in oxidized cholesterol-modified erythrocytes (65–68% of the inhibition) than in the oxidized control (~ 48% of the inhibition) group.

The results shown in Fig. 5 depict positive correlations between PS externalization (measured as percentage of annexin V-labeled cells) and the percentages of phagocytosis-positive cells (expressed as percentage of positive macrophages) in control and cholesterol-modified erythrocytes, always showing a 2.5-fold higher erythrocyte engulfment in the latter group for identical amounts of PS externalized.

We next determined whether *t*-BuOOH affected APLT activity. The data shown in Fig. 6 indicate that the increases in *t*-BuOOH concentrations produced a progressive inhibition of APLT activity in control erythrocytes, with an IC_{50} value estimated at 0.4 mM *t*-BuOOH. Table 1 shows that

Table 1
Changes in aminophospholipid translocase (APLT) activity with respect to MDA contents and isotonic stability observed in control and cholesterol-modified erythrocytes exposed to *t*-BuOOH

Erythrocytes	APLT activity (pmol NBD-PS/ min • mg Hb)	MDA contents (pmol /mg Hb)	Isotonic stability (% hemolysis)
Control			
–*t*-BuOOH	7.04 ± 1.01	0.076 ± 0.020	5.8 ± 2.2
+0.5 mM *t*-BuOOH	2.94 ± 0.69*	0.610 ± 0.056*	18.7 ± 6.7*
Enriched			
–*t*-BuOOH	4.99 ± 1.43†	0.081 ± 0.005	7.5 ± 0.4
+0.5 mM *t*-BuOOH	3.10 ± 0.57*	1.207 ± 0.478*	29.3 ± 6.7*
Depleted			
–*t*-BuOOH	4.21 ± 1.12†	0.078 ± 0.002	3.0 ± 0.9
+0.25 mM *t*-BuOOH	3.59 ± 0.47*	0.525 ± 0.088*	17.4 ± 3.5*

Enzyme activity is expressed as NBD-PS internalized (pmol/min . mg Hb), MDA contents as pmol/mg Hb, and isotonic stability as % hemolysis in each erythrocyte sample assayed as indicated under Materials and methods. Each determination is the mean ± SD of six independent assays. Comparisons are between erythrocytes not oxidized (–*t*-BuOOH) and oxidized (+*t*-BuOOH) for each erythrocyte type. Differences were considered significant at $^{*}p < 0.001$. In nonoxidized erythrocytes, the differences were significant in each cholesterol-modified erythrocyte group with respect to the control at $^{\dagger}p < 0.01$.

the modification of cholesterol contents produces losses of APLT activity of 29% in the cholesterol-enriched group and of about 40% in the cholesterol-depleted erythrocytes. Oxidative stress also produced significant decreases in APLT activity in all RBC groups: 15% in cholesterol-depleted erythrocytes exposed to 0.25 mM oxidant concentration, and 38% in cholesterol-enriched and 58% in control erythrocytes treated with 0.5 mM *t*-BuOOH. APLT activity in cholesterol-depleted erythrocytes was almost undetectable after oxidation with 0.5 mM *t*-BuOOH (data not shown). The impairment of APLT was accompanied by external expression of PS at the erythrocyte membrane surface (Fig. 7), with significant externalization of the aminophospholipid being found only when APLT activity was inhibited by 50%.

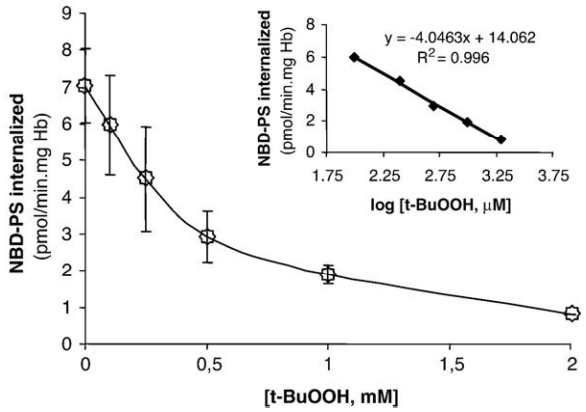

Fig. 6. Effects of *t*-BuOOH on aminophospholipid translocase from control erythrocytes. APLT activity, measured as indicated under Materials and methods, was expressed as picomoles NBD-PS internalized per minute and milligram Hb. Data are means ± SD of six independent assays.

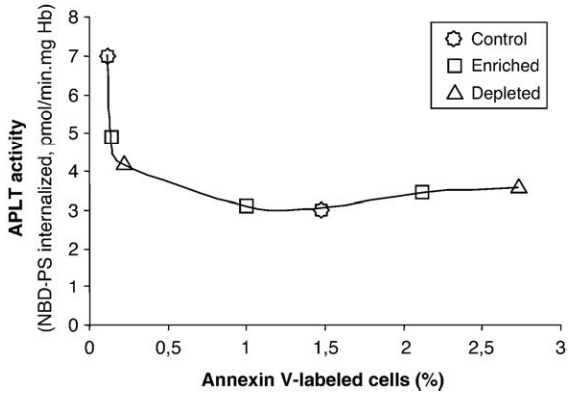

Fig. 7. Influence of APLT activity on the external expression of PS (measured as % of annexin V-labeled cells) in the erythrocyte membrane. Each point is the mean of three separate assays performed in control, cholesterol-enriched, or cholesterol-depleted erythrocytes treated with progressive (0–0.5 mM) *t*-BuOOH concentrations.

The decrease in the isotonic stability (expressed as increase in the percentage of hemolysis) of oxidized erythrocytes revealed a positive correlation with MDA equivalent contents (Fig. 8A). A hemolysis degree of 50% was found at 2.9 pmol MDA equivalents/mg Hb. Fig. 8B shows the morphological changes observed in control and cholesterol-modified erythrocytes before and after exposure to *t*-BuOOH. In nonoxidized erythrocytes, about 10% of the controls showed more than one score at their surface. These alterations increased significantly in cholesterol-modified erythrocytes (1.5-fold in the cholesterol-enriched and 3.2-fold in cholesterol-depleted groups). Cholesterol depletion rendered RBCs approximately 2-fold more sensitive to the morphological changes induced by oxidative stress compared to the other erythrocyte groups, because 50% of the population of these RBCs changed their morphology at 0.7 mM *t*-BuOOH, whereas similar changes were detected in control and cholesterol-enriched erythrocytes at 1.5 mM oxidant. Moreover, a good correlation between

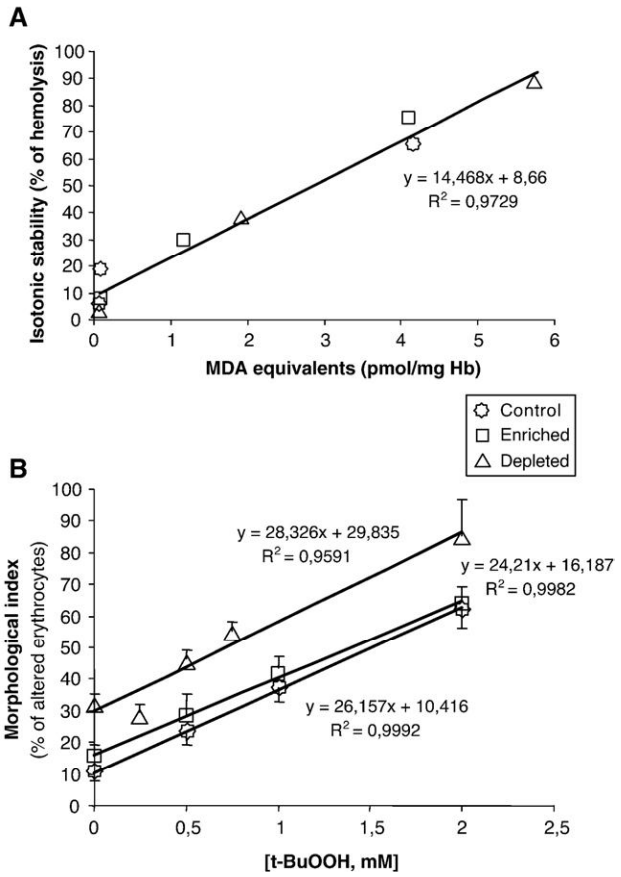

Fig. 8. Analysis of isotonic stability and morphology in erythrocytes exposed to oxidative stress. (A) Relationship between isotonic stability and MDA equivalent contents generated in oxidized erythrocytes. Each point is the mean of three individual determinations performed in control, cholesterol-enriched, or cholesterol-depleted erythrocytes exposed to progressive (0–0.5 mM) *t*-BuOOH concentrations. (B) Morphological index in control, cholesterol-enriched, or cholesterol-depleted erythrocytes after oxidation by various *t*-BuOOH concentrations. Results, given as percentage of altered erythrocytes as indicated under Materials and methods, are means ± SD of five separate determinations.

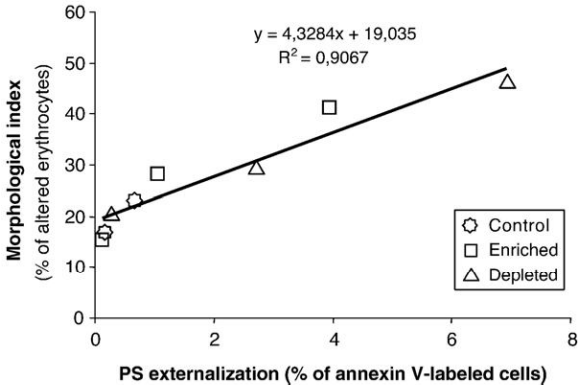

Fig. 9. Effect of the external expression of PS on the morphological changes observed in erythrocytes. Each point represents the mean of three separate determinations performed in control, cholesterol-enriched, or cholesterol-depleted erythrocytes exposed to progressive (0–0.5 mM) *t*-BuOOH concentrations.

altered erythrocyte morphology and PS exposure at the membrane surface was found (Fig. 9).

Discussion

Many membrane functions are perturbed when oxygen-derived and other radicals induce the peroxidation of lipids and the oxidative modification of proteins [38]. The susceptibility of membranes to peroxidation is affected by the nature and physical state of the lipid bilayer [28,29]. Earlier studies by us have provided evidence that modification of cholesterol levels in the membrane of rabbit erythrocytes, above or below those found in the native membrane, has stimulating effects on ROS-induced peroxidation, increasing oxidative damage in treated cells [30], which is blocked in the presence of flavonoid compounds [33]. However, the effect of oxidative damage on asymmetric phospholipid distribution in erythrocytes with different cholesterol contents and its repercussion on cell viability have not yet been analyzed. In order to elucidate this issue, here we modified the cholesterol contents in rabbit erythrocytes, observing an enhanced PS externalization, with an increased clearance of oxidized compared to nonoxidized erythrocytes. On the basis of the analysis of APLT activity, we propose that PS externalization would in part be a consequence of the decrease in APLT activity induced by oxidative stress, such enzyme activity also depending on membrane cholesterol contents.

Structural changes in erythrocytes after the manipulation of cholesterol levels

The modification of membrane cholesterol contents led to a significant exposure of PS at the erythrocyte surface, with an almost identical redistribution (about 5% of PS externalized) after both the enrichment and the depletion of cholesterol, which may be ascribed in part to the similar losses in APLT activity detected in both cholesterol-modified erythrocyte groups. The significant inactivation of APLT after cholesterol modification could be due to physical alterations in the

environment of the enzyme or to a negative regulation through caspase 3. We have previously described that extensive removal of cholesterol by MBCD results in a remarkable increase in the fluidity of the deep lipid core and membrane surface in rabbit erythrocytes, whereas cholesterol enrichment produces enhanced microviscosity in the lipid bilayer [30]. It is well known that fluidity could contribute to changes in protein conformation, protein distribution in the membrane, and/or the function of some membrane-bound enzymes [39,40], including APLT. Recently, it has been reported that PS externalization in early chondrocyte hypertrophy is dependent on a decrease in APLT activity and an increase in phospholipid scramblase activity, through a decrease in membrane cholesterol associated with a reorganization of membrane raft microdomains [41]. Changes in the asymmetric distribution of aminophospholipids have also been found after cholesterol incorporation in sheep platelet plasma membranes and liposomes, with a progressive exposure of these lipids to the outer leaflet of the lipid bilayers [31].

The exposure of PS to the extracellular surface of erythrocytes subsequently stimulates the clearance of these erythrocytes from peripheral blood by phagocytosis [15]. Thus, the erythrophagocytosis assay can also be used as a sensitive probe to detect losses of normal PS asymmetry in intact erythrocytes. In our case, the uptake of nonoxidized erythrocytes by mouse macrophages was found to be enhanced to similar extents after cholesterol enrichment or depletion, which is well correlated with the increased externalization of PS and decreased APLT activity observed in both cases. Meurs et al. [42] also found a reduction in the life span of erythrocytes from SR-BI-deficient mice with high cholesterol contents. They explained this as being due to a significant reduction in the deformability of circulating erythrocytes and increased osmotic fragility.

Modification of cholesterol contents kept RBCs almost intact (> 99% cell viability according to SSC/FSC scatter analysis by flow cytometry, and a degree of hemolysis lower than 2.5%). Analysis of physical parameters in nonoxidized erythrocytes by flow cytometry revealed that viable cells had higher FSC and higher SSC values those found in cell debris, which corresponds to cells with a major discocyte morphology. Cholesterol depletion produced more marked changes in cell shape than the enrichment process, as observed with flow cytometry analysis and morphological studies, which were associated with patent transformations of discocyte forms to echinocytes. These morphological changes were accompanied by the PS externalization observed during the cholesterol-modification processes, in agreement with the data of Wolfs et al., who reported that agents that cause RBCs to change to echinocyte forms also increase the rate of PS exposure at the membrane surface [20]. The formation of echinocytes also leads to a disruption of the interactions between the lipid bilayer and the cytoskeleton [21], which in itself might contribute to an increase in the rate of lipid scrambling. Changes in the structural and chemical composition of erythrocyte membranes, with alterations in cellular shape, have also been reported in human erythrocytes exposed to α-, β-, and γ-cyclodextrins [43].

Oxidative damage in cholesterol-modified erythrocytes

Oxidative damage to erythrocytes has become a key term to explain destructive events in many hematological disorders or in aged cells. It has been suggested that oxidative stress could, directly or indirectly, be the factor responsible for membrane alterations, which would ultimately cause the observed physiological events, such as imbalanced hemostasis and vaso-occlusive crisis in altered cells or removal of such cells from the bloodstream [44,45].

In our study, the asymmetric phospholipid distribution observed in erythrocyte membrane with different cholesterol contents, analyzed by phospholipase A_2 digestion, revealed a significant redistribution of aminophospholipids only after treatment with *t*-BuOOH. PS exposure at the membrane surface was always higher than that found in PE externalization in all oxidized erythrocyte groups. PS externalization was maximum in the cholesterol-depleted group, in accordance with the greater inactivation observed in APLT. Oxidized cholesterol-enriched erythrocytes showed the smallest extent of PS redistribution, even with respect to oxidized control erythrocytes, in agreement with the minimal inactivation detected in APLT. It could be speculated that the cholesterol enrichment in the membrane would in part protect the enzyme against oxidation as the result of a hindrance in ROS propagation in a rigidified environment. This aminophospholipid expression was similar to those found in oxidized RBCs due to the presence of phenylhydrazine [45] or copper(II) ascorbate [46]. The loss of this asymmetric distribution in RBCs has also been reported to occur in processes related to high levels of oxidative stress, such as the hemolytic anemia associated with sickle cell diseases [35] or in aged [47] or banked [34] erythrocytes. Furthermore, these changes are analogous to the events occurring in apoptotic cells [13,22].

Treatment of control and cholesterol-modified erythrocytes with *t*-BuOOH resulted in the binding of FITC-labeled annexin V, an anticoagulant protein that binds to PS-containing phospholipid surfaces, to the cells in an oxidant concentration-dependent manner. Cholesterol depletion in the erythrocyte membrane significantly increased the binding of FITC–annexin V to oxidized cells. This annexin V binding to *t*-BuOOH-stressed erythrocytes could not be attributed solely to PS externalization, because annexin V also binds to negatively charged phospholipids and to aldehyde–lipid adducts [48]. However, PE does not seem to contribute significantly to annexin V binding to the cell membrane because non-oxidized erythrocytes exposed to FITC-labeled annexin V showed minimal fluorescent labeling (< 0.2%), even though at least 11% of all PE was located in the membrane outer monolayer of these nonoxidized erythrocytes. The exponential increase noted between the binding of fluorescently labeled annexin V and MDA equivalents or ROS contents in the erythrocytes (Fig. 3) supports the involvement of oxidative stress in PS externalization, corroborating the redistribution of PS detected by phospholipase A_2 digestion in all oxidized RBCs.

External expression of PS is required for recognition of apoptotic or abnormal cells by macrophages and other phagocytes [15,49]. Our data support these findings, because

incubation of oxidized (with 2 mM *t*-BuOOH) control erythrocytes with annexin V inhibited about 70% of their uptake by macrophages. Moreover, we observed that erythrophagocytosis was increased upon modifying membrane cholesterol contents and that this seemed to be PS externalization-dependent, as suggested by the positive correlations found between annexin V-labeled cells and positive macrophages. *t*-BuOOH-induced stress resulted in an increase in the phagocytosis of the different erythrocyte groups in an oxidant dose-dependent manner, maximal effects being observed after cholesterol depletion. Caspase 3 seems to play a role in such PS externalization, because erythrophagocytosis was blocked when RBCs were incubated with the caspase 3 inhibitor Ac-DEVD-CHO. These findings are in accordance with those reported in oxidized human erythrocytes [7]. Thus, the caspase 3 activation induced by oxidative stress [6,7], and probably by cholesterol modification, could be associated with the impaired APLT activity detected. As proposed by Mandal et al. [6,7], caspase 3 activation could negatively regulate APLT activity through direct proteolytic cleavage of the flippase or through an indirect modulatory role involving intermediate regulators of APLT. However, we cannot exclude the concomitant induction of recognition signals other than PS, such as the degraded band 3 [50] and other ligands that appear on the cell surface after loss of the phospholipid asymmetry [49], especially in cholesterol-modified erythrocytes, where the presence of annexin V only partially blocked erythrocyte uptake.

The main requirement for PS externalization in abnormal cells is the inhibition of APLT because even if scramblase is activated, an active APLT would rapidly transport PS from the outer to the inner monolayer [9,10]. Here we found that PS externalization was correlated with an impaired activity of APLT (Fig. 7 and additional data not shown), although the assessment of a causal link between APLT inactivation and PS externalization requires further study. The enzyme is inactivated in a *t*-BuOOH concentration-dependent manner (Fig. 6), indicating that APLT contains an SH group(s) that is critical for function and that is hypersensitive to *tert*-butoxyl radicals or to its oxidation products formed in the oxidative process, as reported earlier [23,24]. Maximal impairment of APLT was found in the oxidized cholesterol-depleted group, probably as a consequence of the enhanced propagation of ROS through the destabilized membrane. The inactivation of APLT alone would probably be insufficient to explain the PS externalization detected in our study because we found progressive increases in PS exposure when almost identical degrees of impairment of the APLT had been reached (Fig. 7). High levels of cytosolic calcium could occur in premorbid RBCs as a result of severe ATP depletion and/or an inhibition of the Ca^{2+} pump ATPase by exposure to *t*-BuOOH [51]. From this study, we envisage that the expression of PS at the erythrocyte membrane surface under oxidative stress could be due at least in part to the impairment detected in APLT, which could in turn be associated with a concomitant activation of Ca^{2+}-dependent phospholipid scramblase as a consequence of elevated cytoplasmic calcium. A significant increase in PS externalization cannot be ruled out because of the oxidation and/or proteolysis of cytoskeletal proteins such as spectrin and band 4.1, which could produce a disruption of PS-skeletal protein interactions [21]. The oxidized PS forms generated by *t*-BuOOH treatment could even facilitate transbilayer movements of aminophospholipids across the plasma membrane [17].

The isotonic stability of different erythrocyte groups was decreased by the oxidative stress, producing cells that were more unstable in the cholesterol-modified groups. The accumulation of oxidized lipids around the aggregated band 3 may lead to the formation of hemolytic holes and leakage sites. These leakage sites induce colloid–osmotic cell lysis through the uptake of solutes and water via these holes [52,53].

The cell morphology of the different erythrocyte groups changed progressively from discocytes to echinocyte forms after exposure to increased *t*-BuOOH concentrations. Depletion of the cholesterol contents in erythrocytes rendered them more sensitive to morphological cellular changes after oxidation, with a positive correlation between the loss of PS asymmetry and the percentages of altered cells (Fig. 9). These morphological changes coincide with those seen in erythrocytes, whose aminophospholipid transport system was inhibited and from which high amounts of PS were externalized [54]. The intercalation of sufficient amounts of aminophospholipids in the outer monolayer of the erythrocyte membrane results in a greater relative expansion of this leaflet, which leads to echinocyte forms [20].

In conclusion, our study provides clear evidence that the modification of cholesterol contents in RBCs promotes an enhanced externalization of PS at their membrane surface, which is consistent with the significant inhibition of APLT and an increased uptake of modified erythrocytes by macrophages. Changes in membrane cholesterol levels have stimulating effects on oxidative stress, which causes increases in PS externalization and inactivation of APLT. PS exposure is positively correlated with the enhanced phagocytosis and increased echinocytosis observed in all oxidized erythrocytes and is especially pronounced after cholesterol depletion. In this sense, it may be speculated that the enhanced exposure of aminophospholipids at the erythrocyte membrane surface produced by cholesterol depletion could be the cause of the higher oxidative damage observed in this erythrocyte group, because mainly at this location the negative polar heads of the aminophospholipids are able to bind iron, whose chelation accelerates the decomposition of *t*-BuOOH [55,56], thus propagating the ROS through this destabilized membrane at higher rate. In agreement with the data reported here and previous results [30,42], we suggest a possible role for membrane cholesterol levels, together with the normal asymmetric location of aminophospholipids in the membrane lipid bilayer, in the sensitivity of RBCs to oxidative stress, which will eventually affect erythrocyte survival. Moreover, our study could provide a possible mechanism by which hypercholesterolemia could increase hypercoagulation in cardiovascular and neurodegenerative disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2007.01.010.

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