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# Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes

Debabrata Mandal, Prasun K. Moitra, Samiran Saha, Joyoti Basu*

*Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Kolkata 700009, India*

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**Abstract The appearance of phosphatidylserine (PS) on the outer surface of red cells is an important signal for their uptake by macrophages. We report for the first time that procaspase 3 present in the anucleated mature human erythrocyte is activated under oxidative stress induced by *t*-butylhydroperoxide leading to impairment of the aminophospholipid translocase, PS externalization and increased erythrophagocytosis. This is the first report linking caspase 3 activation to inhibition of flippase activity and uptake of red cells by macrophages.** 

*Key words:* Caspase 3; Human erythrocyte; Phospholipid asymmetry; Phosphatidylserine; Aminophospholipid translocase; Phagocytosis

## 1. Introduction

Normal circulating blood cells exhibit an asymmetric distribution of phospholipids in the membrane where phosphatidylserine (PS) and phosphatidylethanolamine (PE) reside in the inner leaflet and phosphatidylcholine (PC) and sphingomyelin are enriched in the outer leaflet [1]. While asymmetry is the rule for normal blood cells, loss of asymmetry is associated with pathologic phenomena in blood cells [2]. The ATP-dependent aminophospholipid translocase transports PS and, to a lesser extent, PE from the outer to the inner leaflet of the plasma membrane against the concentration gradient, thereby compensating for any escape of PS or PE to the outer leaflet [3–5]. In contrast to the translocase, calcium-dependent bidirectional movement of phospholipids across the membrane, termed scrambling, shows no phospholipid specificity [6–8]. PS externalization has been linked to the anemia associated with the human thalassemias [9,10] and with hyperglycemia [11]. It has also been suggested that aminophospholipid translocase activity diminishes during in vivo and artificial aging of human erythrocytes as a consequence of oxidative stress [12].

*Corresponding author. Fax: (91)-33-3506790.
*E-mail address:* joyoti@bosemain.boseinst.ac.in (J. Basu).

*Abbreviations:* PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NBD-PS, 1-palmitoyl-2-[6-[7(nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-caproyl]-*sn*-glycerol-3-phosphoserine; *t*-BHP, *t*-butylhydroperoxide; Z-DEVD-FMK, benzyloxycarbonyl-Asp($OCH_3$)-Glu($OCH_3$)-Val-Asp($OCH_3$)-fluoromethylketone; Ac, acetyl; *p*NA, *p*-nitroanilide

Apoptosis is a mode of programmed cell death that is under molecular control [13]. Senescence of erythrocytes and apoptosis of nucleated cells share the feature of externalization of PS. Caspases are aspartate-specific cysteine proteinases that exist as latent zymogens, but once activated by apoptotic signals, they promote apoptosis by specific limited proteolysis of key cellular substrates [14]. Caspase 3 is synthesized as a 32 kDa zymogen that is processed to mature 20/17 kDa and 12 kDa subunits by cleavage at Asp9, Asp28 and Asp175 [15,16].

We have asked the question whether caspase 3 is present in erythrocytes and whether it is involved in the process of PS externalization and erythrophagocytosis in oxidatively stressed erythrocytes. Our results demonstrate that procaspase 3 is present in mature erythrocytes. We demonstrate for the first time in anucleated cells that the process of PS externalization under oxidative stress is dependent on the activation of caspase 3 which is associated with impairment of aminophospholipid flippase activity.

## 2. Materials and methods

### 2.1. Materials

The caspase 3 inhibitor Z-DEVD-FMK and acetyl-DEVD-*p*-nitroanilide (Ac-DEVD-*p*NA) were products of CN Biosciences (San Diego, CA, USA). Anti-caspase 3 antibody was from BD Pharmingen (USA). The Annexin-fluos kit was from Roche Molecular Biochemicals (Mannheim, Germany); *t*-butylhydroperoxide (*t*-BHP) was a product of Sigma Chemical Co. (St. Louis, MO, USA). 1-Palmitoyl-2-[6-[7(nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-caproyl]-*sn*-glycerol-3-phosphoserine (NBD-PS) was from Avanti Polar Lipids (Birmingham, AL, USA).

### 2.2. Collection of blood and treatment with t-BHP

Blood was collected from normal healthy donors after informed consent, erythrocytes were separated from whole blood on Ficoll, washed in phosphate-buffered saline (PBS), treated at 5% hematocrit with *t*-BHP at 37°C, aliquots were taken at different time intervals and pelleted. 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 three times in a five-fold volume of PBS, and kept on ice for further analysis.

### 2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis of caspase 3

Erythrocytes were osmotically lysed in hypotonic solution and the lysate was incubated overnight with rabbit anti-caspase 3 antibody at 4°C. Protein A/G agarose was added and incubated for 1 h at room temperature and the immunoprecipitate was washed, denatured and separated by SDS–PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-caspase 3 antibody followed by alkaline phosphatase-linked goat anti-rabbit secondary antibody and finally visualized using NBT/BCIP color developing reagent.

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*2.4. Analysis of caspase 3 activity*

DEVD-dependent protease activity was determined using Ac-DEVD-*p*NA as substrate and the immunosorbent caspase 3 activity assay kit from Roche Molecular Biochemicals. Briefly, after treatment of erythrocytes with *t*-BHP, lysates were prepared and caspase 3 was captured from the lysate obtained from 100 μl erythrocytes (5% hematocrit) in microplates coated with anti-caspase 3 monoclonal antibody. Following washing of the plates, Ac-DEVD-*p*NA was added and the released *p*NA was determined spectrophotometrically at 405 nm. A *p*NA calibration curve was plotted from a *p*NA stock solution and the caspase 3 activity was measured relative to this curve.

*2.5. Erythrophagocytosis assay*

Murine macrophage J774A.1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Phagocytosis assays were carried out as described by Pradhan et al. [17]. Briefly, erythrocytes ($2\times10^6$ cells) were layered over macrophages ($5\times10^5$ cells) on a coverslip and incubated at 37°C for 1 h. Non-adherent cells were removed by gentle washing with PBS and surface-bound erythrocytes were lysed by treatment with 140 mM $NH_4Cl$/17 mM Tris–HCl, pH 7.6 for 5 min. Cells were stained with diaminobenzidine for staining erythrocytes, and counterstained with Giemsa stain for staining macrophages. Phagocytosis was calculated in terms of the percentage of macrophages that ingested one or more erythrocytes. *t*-BHP treatment was done for 60 min at 37°C prior to erythrophagocytosis assays.

*2.6. Preparation of liposomes*

Liposomes composed of brain PS, egg PC and cholesterol (C) at a molar ratio of 1:1:1 or of PC and C at a molar ratio of 2:1 were prepared as described by Sambrano and Steinberg [18]. Lipids and butylated hydroxytoluene (20 μM) were dried under a stream of nitrogen, rehydrated in 40 mM Tris–HCl/140 mM NaCl to a final total lipid concentration of 1.5 mM, mixed by vortexing and subsequently sonicated in a bath-type sonicator under a nitrogen stream until clear.

*2.7. Determination of aminophospholipid translocase activity*

Flippase activity was assessed by the extent of fluorescent phospholipids retained in the plasma membrane after back-extraction with bovine serum albumin (BSA) which extracts only those exogenously added fluorescent phospholipids and not endogenous phospholipids from the outer monolayer [19]. Briefly, prior to labeling, erythrocytes were incubated with 5 mM diisopropyl-fluorophosphate in order to prevent degradation of NBD-PS. NBD-PS was added to a final concentration of 1 μM to erythrocytes, incubated on ice for 5 min in buffer A containing 5 mM HEPES, pH 7.5, 14 mM NaCl, 5 mM KCl, 1 mM $Mg^{2+}$ and 10 mM glucose. Cells were washed and resuspended in 2 ml of buffer A. 100 μl aliquots were removed at different times intervals and added to 1 ml of buffer A containing 1% fatty acid-free BSA in order to remove NBD-PS present in the outer leaflet. Fluorescence associated with the supernatant was measured in a spectrofluorimeter (excitation wavelength: 472 nm, emission wavelength: 534 nm).

*2.8. Labeling with annexin-fluos and flow cytometric analysis*

Labeling with annexin-fluos to detect PS exposure on red cells was performed using the Annexin-fluos kit according to the manufacturer's instructions. Data acquisition was performed on a Becton Dickinson FACS Calibur flow cytometer and analysis was done with Cell Quest software. 10 000 events per sample were acquired to ensure adequate mean fluorescence levels. 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.

*2.9. Statistical analysis*

Data obtained from independent experiments are represented as mean ± S.D. and were compared by Student's *t*-test. Differences were considered significant at $P<0.05$.

## 3. Results

*3.1. Oxidative stress activates caspase 3 in human erythrocytes*

Erythrocytes were treated with different concentrations of *t*-BHP for different times. The appearance of the 20 kDa form after *t*-BHP (3 mM) treatment in a time-dependent manner was considered to be an index of activation of caspase 3 (Fig. 1A). Caspase activation was further confirmed by measuring the cleavage of the caspase 3-specific substrate Ac-DEVD-*p*NA. After treatment of erythrocytes with 3 mM *t*-BHP for 60 min at 37°C, caspase 3 was captured from lysates in microtiter plates coated with anti-caspase 3 antibody. A time-dependent release of *p*NA was observed (Fig. 1B). DEVD-*p*NA-hydrolyzing activity was maximal by 2 h. In contrast, lysate from erythrocytes incubated at 37°C in the absence of *t*-BHP for 20 min did not show any DEVD-*p*NA-hydrolyzing activity.

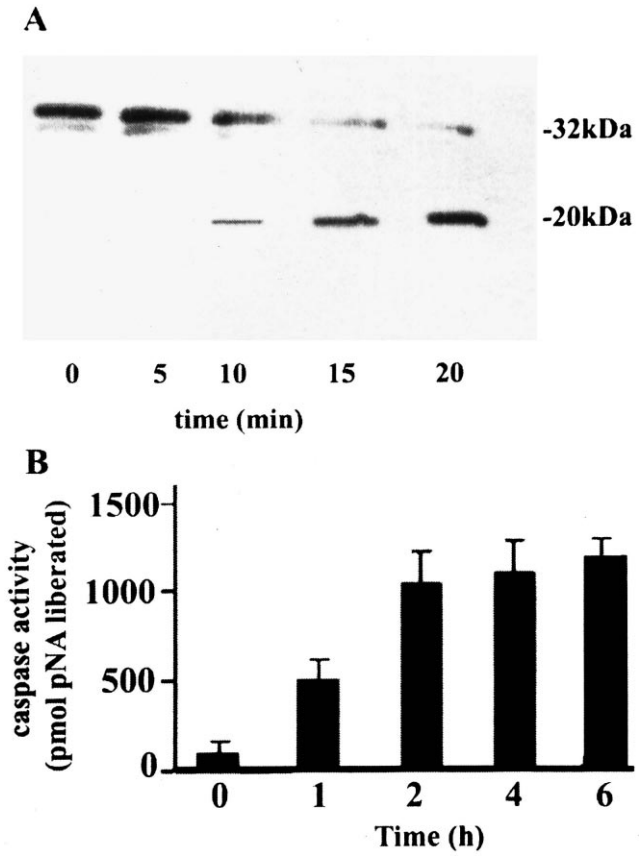


Fig. 1. Caspase 3 activation by *t*-BHP treatment. Red blood cells were treated with 3 mM *t*-BHP at 37°C, harvested at different time periods and lysed. A: Lysates were immunoprecipitated with anti-caspase 3 antibody, and analyzed for caspase 3 by Western blotting as described in Section 2. The panel is representative of three separate experiments. B: Caspase 3 was captured from lysates (obtained after treatment with 3 mM *t*-BHP for 60 min) on to microtiter plates coated with anti-caspase 3 antibody. Ac-DEVD-*p*NA (100 μM) was added to each well and the release of *p*NA was measured spectrophotometrically at 405 nm at the indicated times. Results represent the mean of five sets of determinations ± S.D.

*3.2. PS externalization governs erythrophagocytosis in t-BHP-stressed erythrocytes*

*t*-BHP-induced stress resulted in an increase in phagocytosis of erythrocytes compared to normal erythrocytes (Table 1). This was largely dependent on the externalization of PS, since coincubation of erythrocytes with PS vesicles led to a competitive inhibition of erythrophagocytosis of *t*-BHP-stressed

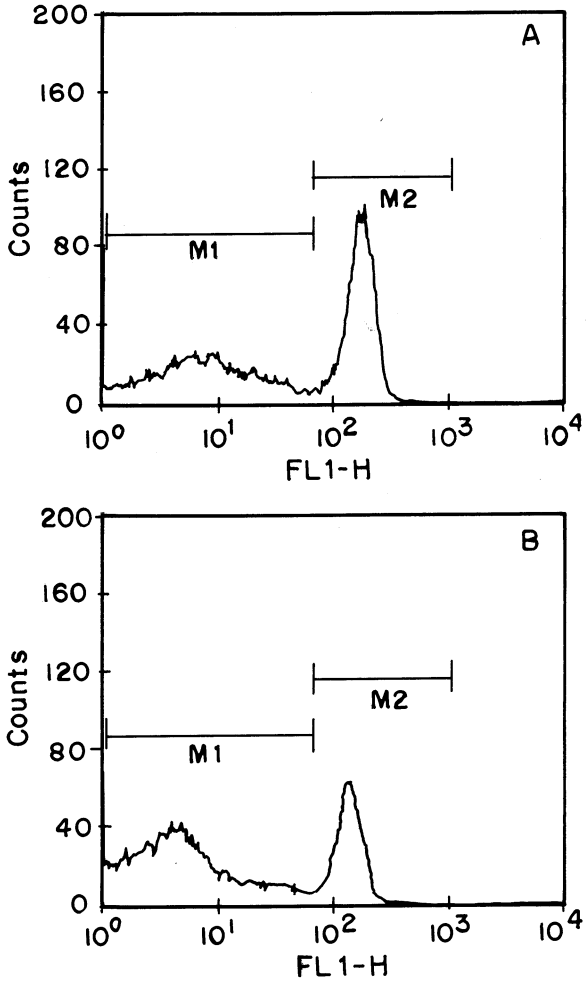


Fig. 2. FACS analysis of annexin-fluos-positive erythrocytes after *t*-BHP treatment. A: Cells treated with 3 mM *t*-BHP. B: Cells pre-incubated with 100 μM Z-DEVD-FMK followed by treatment with 3 mM *t*-BHP for 60 min. The relative number of cells found in the region marked by M2 was defined as the percentage of annexin-fluos-positive red blood cells.

erythrocytes (Table 1) in a dose-dependent manner, whereas PC vesicles (containing no PS) had no effect.

### 3.3. *t-BHP-mediated PS externalization*

Annexin V binding to PS is a widely used index of PS externalization. The percentage of cells binding annexin-fluos was measured by flow cytometry. In six separate determinations, it was observed that red cells treated with 1 mM and 3 mM *t*-BHP for 60 min had $5 \pm 0.5\%$ and $60 \pm 5\%$ (means ± S.D.) of the cells labeled with annexin-fluos respectively. Untreated red cells had $< 0.5\%$ of the population labeled with annexin-fluos after incubation in buffer.

### 3.4. *Inhibition of caspase 3 with Z-DEVD-FMK blocks PS externalization and erythrophagocytosis in t-BHP-stressed erythrocytes*

The involvement of caspase 3 in the process of phagocytosis of red cells induced by *t*-BHP was supported by the observation that pretreatment of the erythrocytes with Z-DEVD-FMK (in 25 mM HEPES, 150 mM NaCl, pH 7.2 for 3 h at 25°C) led to a significant decrease of *t*-BHP-induced phagocytosis of red cells (Table 1). Maximum inhibition of erythrophagocytosis was achieved when cells were pretreated with the inhibitor at a concentration of 100 μM for 3 h prior to oxidative stress. In harmony with this was the observation that treatment with Z-DEVD-FMK under similar conditions led to an approximately 50% decrease (from $60 \pm 6\%$ to $29 \pm 3\%$, six determinations) of PS externalization as visualized by flow cytometry. Fig. 2 shows a representative flow cytometric analysis of PS externalization in red blood cells treated with 3 mM *t*-BHP without and with pretreatment with 100 μM Z-DEVD-FMK for 3 h.

### 3.5. *Oxidative stress-induced impairment of aminophospholipid translocase activity is dependent on caspase 3*

The translocation of PS from the outer to the inner leaflet was measured after loading cells with fluorescent NBD-PS. It was observed that PS externalization in *t*-BHP-stressed red cells correlated with an impairment of aminophospholipid translocase activity (Fig. 3) which could be blocked when cells were treated with Z-DEVD-FMK prior to treatment with *t*-BHP suggesting that caspase 3 was involved in the process of loss of PS asymmetry in oxidatively stressed red cells through its ability to impair the activity of the aminophospholipid translocase.

## 4. Discussion

Since apoptosis in nucleated cells and erythrocyte senescence share the common feature of PS externalization, we explored the probable presence of caspase(s) in mature human erythrocytes and their involvement in disruption of erythrocyte PS asymmetry. Recent studies have demonstrated that

Table 1
Uptake of oxidatively stressed erythrocytes by macrophages

| Group | Positive macrophages (%) |
|---|---|
| Normal | $7 \pm 1$ |
| Normal+*t*-BHP (1 mM)[a] | $15 \pm 3$ |
| Normal+*t*-BHP (3 mM) | $48 \pm 6$ |
| Normal+*t*-BHP (3 mM)+PS (2 nmol) | $30 \pm 5$ |
| Normal+*t*-BHP (3 mM)+PS (4 nmol) | $22 \pm 5$ |
| Normal+*t*-BHP (3 mM)+PS (8 nmol) | $12 \pm 3$ |
| Normal+*t*-BHP (3 mM)+PS (16 nmol) | $9 \pm 2$ |
| Normal+*t*-BHP (3 mM)+PC (16 nmol) | $45 \pm 7$ |
| Normal+*t*-BHP (3 mM)+Z-DEVD-FMK (50 μM) | $25 \pm 5$ |
| Normal+*t*-BHP (3 mM)+Z-DEVD-FMK (100 μM) | $15 \pm 3$ |

Results represent the mean ± S.D. of six separate determinations.
[a]Treatment with *t*-BHP was carried out at 37°C for 60 min.

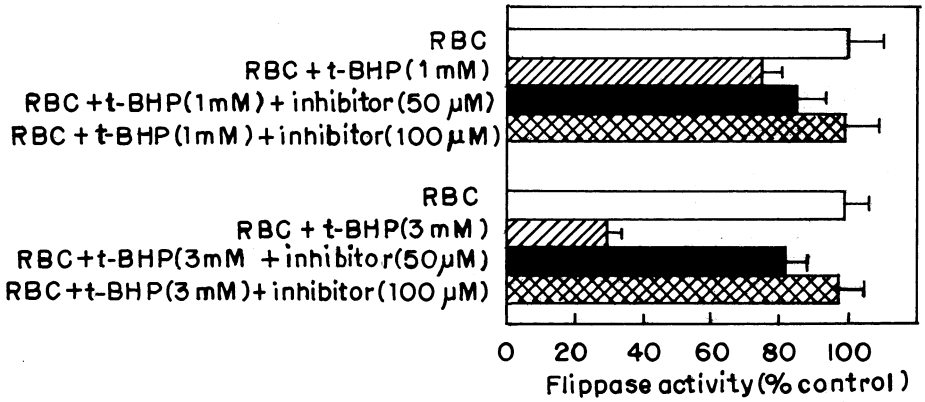


Fig. 3. Flippase activity in *t*-BHP-treated erythrocytes. Cells were incubated at room temperature for 3 h without or with Z-DEVD-FMK (caspase 3 inhibitor) followed by incubation without or with *t*-BHP for 60 min at 37°C as described in Section 2. Aminophospholipid translocase activity was measured after labeling with NBD-PS as described. NBD-PS internalization after 60 min in untreated red cells (i.e. without incubation) was taken to be 100%. Bars represent the mean ± S.D. of six separate determinations.

anucleated cells such as platelets contain functional proapoptotic caspases such as caspase 3 and 9 [20]. The results reported here offer some potentially important insights into the process of erythrophagocytosis of oxidatively stressed erythrocytes. We observe that procaspase 3 present in the mature human erythrocyte undergoes cleavage from the 32 kDa zymogen to the 20 kDa active form in oxidatively stressed erythrocytes. Caspase 3 activation was confirmed by measuring the ability of lysates derived from *t*-BHP-treated red cells to cleave the caspase 3 substrate Ac-DEVD-*p*NA. Incubation of oxidatively stressed red cells with annexin-fluos followed by flow cytometry suggested that *t*-BHP-induced oxidative stress to red cells leads to externalization of PS which could be blocked in part by pretreatment with the caspase 3 inhibitor Z-DEVD-FMK. In the light of recent observations that annexin V can also bind to membrane products of lipid peroxidation [21], the enhanced binding of annexin V to *t*-BHP-stressed red cells could not, however, be attributed solely to PS externalization. Nevertheless, the role of caspase 3-mediated PS externalization in erythrophagocytosis was supported by our observations that *t*-BHP-induced erythrophagocytosis could be blocked by competition with PS-containing liposomes or when red cells were incubated with the caspase 3 inhibitor Z-DEVD-FMK prior to treatment with *t*-BHP. We further observed that loss of the asymmetric distribution of PS was causally linked to impaired activity of the aminophospholipid translocase. Loss of aminophospholipid translocase activity could be blocked when red cells were incubated with the caspase 3 inhibitor Z-DEVD-FMK prior to treatment with *t*-BHP. We propose that caspase 3 activation plays a crucial role in loss of PS asymmetry and erythrocyte phagocytosis under oxidative stress. We envision a process wherein caspase 3 activation negatively regulates aminophospholipid translocase either through direct proteolytic cleavage of the aminophospholipid translocase or, more likely, through an indirect modulatory role involving intermediate regulators of the flippase. However, the fact that neither *t*-BHP-induced erythrophagocytosis nor PS externalization could be completely reversed by Z-DEVD-FMK suggests that caspase-mediated loss of aminophospholipid translocase activity is one of the factors, but not the only one governing these processes. This is the first demonstration of the critical role of caspase 3 in potentially regulating the life span of the circulating mature human erythrocyte by impairing the activity of the aminophospholipid translocase.

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