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Role of membrane oxidation in controlling the activity of human group IIa secretory phospholipase A_2 toward apoptotic lymphoma cells

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ABSTRACT

The membranes of healthy lymphocytes normally resist hydrolysis by secretory phospholipase A_2. However, they become susceptible during the process of apoptosis. Previous experiments have demonstrated the importance of certain physical changes to the membrane during cell death such as a reduction in membrane lipid order and exposure of phosphatidylserine on the membrane surface. Nevertheless, those investigations also showed that at least one additional factor was required for rapid hydrolysis by the human group IIa phospholipase isozyme. This study was designed to test the possibility that oxidation of membrane lipids is the additional factor. Flow cytometry and confocal microscopy with a fluorescent probe of oxidative potential suggested that oxidation of the plasma membrane occurs during apoptosis stimulated by thapsigargin. When oxidative potential was high, the activity of human group IIa secretory phospholipase A_2 was enhanced 30- to 100-fold compared to that observed with conditions sufficient for maximal hydrolysis by other secretory phospholipase A_2 isoforms. Direct oxidation of cell membranes with either of two oxidizing agents also stimulated hydrolysis by secretory phospholipase A_2. Both oxidizers caused externalization of phosphatidylserine, but a change in lipid order did not always occur. These results demonstrated that membrane oxidation strongly stimulates human group IIa secretory phospholipase A_2 activity toward apoptotic cells. Interestingly, the change in membrane order, previously thought to be imperative for high rates of hydrolysis, was not required when membrane lipids were oxidized. Whether phosphatidylserine exposure is still necessary with oxidation remains unresolved since the two events could not be deconvoluted.

1. Introduction

Recently, we have focused on the relationship between apoptosis and the activity of various isoforms of $sPLA_2$. Numerous studies have identified two physical changes as being relevant to the action of $sPLA_2$ during cell death: 1) an increase in the presence of anionic phospholipids (generally phosphatidylserine, PS) in the outer leaflet of the membrane [1–7] and 2) a decrease in membrane lipid order reflecting diminished strength of interactions among adjacent phospholipids [5–11]. In addition, studies with calcium ionophore-stimulated death indicated that a third membrane alteration must be essential for the human group IIa isoform (hGIIa) of the enzyme [7,12]. Even though ionophore-stimulated calcium uptake caused immediate full exposure of PS and maximal reduction in lipid order, hGIIa $sPLA_2$ did not hydrolyze the cell membrane. These observations strongly contrasted the response of the enzyme to artificial membranes composed entirely of anionic phospholipids [3,4,7,12–14]. Furthermore, a different isoform, hGX, hydrolyzed ionophore-treated cells at a greater rate than apoptotic cells and appeared not to require a third factor [7].

A recent publication reported that some apoptotic inducers produce a subpopulation of cells with slight permeability to propidium iodide [12]. This modest permeability correlated with increased susceptibility to the hGIIa isozyme. That study suggested that plasma membrane oxidation could be a candidate for the third membrane alteration mentioned above [12]. This hypothesis is supported by three observations. First, cytochrome c release during apoptosis causes lipid oxidation [15–20]. Second, lipid oxidation enhances the permeability of artificial bilayers [19,21–23]. Third, membranes containing oxidized lipids are more susceptible to hydrolysis by the hGIIa isoform of $sPLA_2$ [24–28].

We have therefore tested the hypothesis that membrane oxidation is the third factor required for a membrane to become susceptible to hydrolysis by hGIIa $sPLA_2$. Accordingly, we assayed lipid oxidation potential of S49 lymphoma cells during death triggered by thapsigargin (TG), an inhibitor of the endoplasmic reticulum Ca^{2+} ATPase that causes ER stress [29–31]. Activity of hGIIa $sPLA_2$ was then assayed to determine if susceptibility correlated with membrane oxidation. Cell membranes were then directly oxidized using *tert*-butyl hydroperoxide (TBHP) or 2,2′-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH, a

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hydrophilic radical initiator), and hydrolytic activity of hGIIa $sPLA_2$ was determined. Externalization of PS and lipid order were also assessed to establish which of these membrane changes is necessary for hGIIa $sPLA_2$ activity.

2. Materials and methods

2.1. Reagents

The hGIIa and snake venom (*Agkistrodon piscivorus piscivorus*) isoforms of $sPLA_2$ were isolated and prepared as described [32,33]. Thapsigargin and NecroX-5™ were obtained from Enzo Life Sciences (Plymouth Meeting, PA). Acrylodan-labeled fatty acid-binding protein (ADIFAB), merocyanine 540 (MC540), 6-palmitoyl-2-[[2-(trimethylammonio)ethyl]methylamino]naphthalene chloride (Patman), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$) were purchased from subsidiaries of Life Technologies (Grand Island, NY). The oxidizers TBHP and AAPH were purchased from Sigma-Aldrich (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI), respectively. Trolox was also obtained from Cayman Chemical.

2.2. General

S49 murine lymphoma cells were cultured in suspension in Dulbecco's Modified Eagle Medium containing 10% heat-inactivated horse serum as described (10% CO_2, 37 °C, [34]). Cells were harvested, washed, and resuspended in a balanced salt buffer (134 mM NaCl, 6.2 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 18 mM HEPES, 13.6 mM glucose, pH 7.4 at 37 °C) to a density of $0.5–2\times10^6$/ml prior to experimentation. Treatment with TG (5 μM), AAPH (3 mM, dissolved in culture medium fresh immediately prior to each experiment), or associated controls was done in culture media. For experiments involving TBHP (0.4 mM), cells were treated after harvesting. All experiments were performed at 37 °C. In all quantitative representations of the data, error bars represent the range or SE ($n>2$).

2.3. Confocal microscopy

Images were collected on an Olympus FluoView FV 300 confocal laser scanning microscope using a 60× oil immersion objective lens. Cells were stained with C11-BODIPY$^{581/591}$ (2 μM final) for at least 1 h in culture prior to treatment with TG or an oxidizer. Two different lasers and emission filter sets were used concurrently to excite and collect fluorescence from both oxidized (488 nm argon laser, 505–525 nm bandpass emission filter, green color in images) and non-oxidized probes (543 nm helium-neon laser, 560–660 nm bandpass emission filter, red color in images). Experimental temperature was maintained throughout imaging using a heated stage.

2.4. Flow cytometry

When assaying oxidative potential, cells were incubated with C11-BODIPY$^{581/591}$ as described above. To assay PS externalization, cells were treated with TG, oxidizing agents, or control vehicle as described above and then stained with annexin V Alexa Fluor® 488 conjugate for 15 min after cell harvesting. Samples were processed in a BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA) with an argon excitation laser (488 nm) and an emission bandpass filter (515–545 nm).

For C11-BODIPY$^{581/591}$, fluorescence histograms of control sample fluorescence were fit to a Gaussian curve. Fluorescence histograms of treated samples were then fit with two Gaussian curves with one constrained to the mean and standard deviation of the corresponding control. The area under the second Gaussian curve normalized to the total fluorescence was reported as the percent of the population staining positive for oxidized C11-BODIPY$^{581/591}$. Data from annexin V samples were gated according to the intensity profile of samples

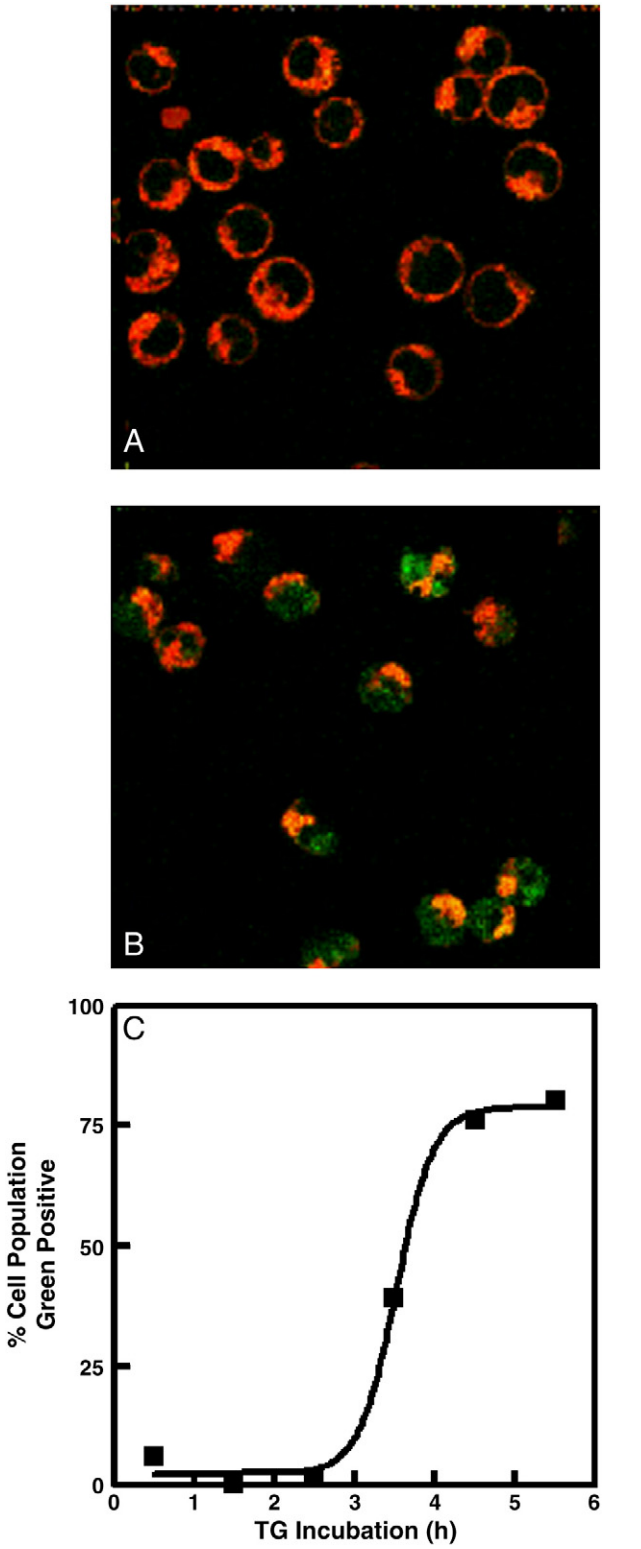

Fig. 1. Plasma membrane oxidation during TG-induced apoptosis. Lipid oxidative potential of the membrane was assessed by confocal microscopy (Panels A, B) and flow cytometry (Panel C) using C11-BODIPY$^{581/591}$. Probes fluorescing green represent those that have been oxidized. Panel A: control cells. Panel B: cells treated with thapsigargin for 4.5 h.