Phosphatidylserine peroxidation/externalization during staurosporine-induced apoptosis in HL-60 cells

Tatsuya Matsura[a], Behice F. Serinkan[a], Jianfei Jiang[a], Valerian E. Kagan[a,b,c,*]

[a] *Department of Environmental and Occupational Health, University of Pittsburgh, 3343 Forbes, Pittsburgh, PA 15260, USA*
[b] *Department of Pharmacology, University of Pittsburgh, 3343 Forbes, Pittsburgh, PA 15260, USA*
[c] *Pittsburgh Cancer Institute, University of Pittsburgh, 3343 Forbes, Pittsburgh, PA 15260, USA*

Received 14 May 2002; revised 13 June 2002; accepted 19 June 2002

First published online 28 June 2002

Edited by Vladimir Skulachev

Abstract Although oxidative stress is commonly associated with apoptosis, its specific role in the execution of the apoptotic program has yet to be described. We hypothesized that catalytic redox interactions between negatively charged phosphatidylserine (PS) and positively charged cytochrome *c* released into the cytosol, along with the production of reactive oxygen species (ROS), results in pronounced oxidation and externalization of PS, and subsequent recognition of apoptotic cells by macrophages. By using staurosporine, a protein kinase inhibitor that does not act as a prooxidant, we were able to induce apoptosis in HL-60 cells without triggering the confounding effects of non-specific oxidation reactions. Through this approach, we demonstrated for the first time that PS underwent a statistically significant and pronounced oxidation at an early stage (2 h) of non-oxidant-induced apoptosis while the most abundant phospholipid, phosphatidylcholine, did not. Glutathione (GSH), the most abundant cytosolic thiol, also remained unoxidized at this time point. Furthermore, PS oxidation and the appearance of cytochrome *c* in the cytosol were concurrent; PS externalization was followed by phagocytosis of apoptotic cells. These findings are compatible with our proposed roles for oxidative PS-dependent signaling during apoptosis and phagocytosis. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Phosphatidylserine externalization; Phosphatidylserine peroxidation; Apoptosis; Cytochrome *c*; Staurosporine; Phagocytosis

1. Introduction

Many different prooxidants such as redox-cycling chemicals, photosensitizers, and UV- and X-irradiation cause apoptosis because of their propensity to induce oxidative stress. These exogenous agents trigger apoptosis through oxidative modification of critical intracellular targets, particularly DNA and mitochondria (reviewed in [1,2]). Once initiated, apoptosis inevitably involves production of reactive oxygen species (ROS) by mitochondria as an intrinsic part of executing of the apoptotic program [3]. This 'endogenous' oxidative stress is mainly caused by the departure of cytochrome *c* from mitochondria, disruption of electron transport, impairment of proton flow and decrease of the mitochondrial transmembrane potential, and excessive generation of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) [4,5]. The role of this 'endogenous' oxidative stress in subsequent stages of the apoptotic program and in signaling is not well understood. In fact, whether it is an unavoidable but useless side effect of apoptosis or a critical feature of the apoptotic cascades through modification of redox-sensitive mechanisms (e.g. mitochondrial permeability transition pore (reviewed in [6]) and caspases [7]) is not quite clear.

Phospholipids and their enzymatic and non-enzymatic oxidation products, such as eicosanoids, lipoxins, and isoprostanes, are known to function as physiologically important signaling molecules (reviewed in [8–10]). It is possible that oxidation-sensitive polyunsaturated phospholipids may participate in signaling during apoptosis. Unfortunately, information on peroxidation of phospholipids during apoptosis is scarce primarily because quantitative assays for oxidation of different classes of phospholipids are not readily available. One major reason for this is a very effective system of remodeling and repair of oxidatively modified phospholipids [11] that interferes with their accurate measurement.

We have recently developed and optimized a technique for quantitative assessment of oxidative stress in different classes of phospholipids in live cells: through metabolic labeling of phospholipids with an oxidation-sensitive fluorescent fatty acid, *cis*-parinaric acid (PnA) [11].

We previously reported that phosphatidylserine (PS) underwent more pronounced oxidation than other phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin during oxidant-induced apoptosis [12]. We hypothesized that PS *oxidation* is associated with its *externalization* on the surface of the plasma membrane during apoptosis [13]. PS externalization is an early, prominent feature of apoptosis [14] and acts as an important recognition signal for phagocytosis of apoptotic cells [15]; on that basis we further speculated that PS *oxidation* may be involved in a signaling pathway responsible for safe clearance of apoptotic cells by macrophages [12,13]. The hypothesis implies that PS is oxidized during execution of the apoptotic program. While our earlier results provided some indirect evidence in favor of this hypothesis, we failed to detect preferential oxidation of PS due to massive oxidation of essentially all types of phospholipids during oxidant-induced apoptosis.

*Corresponding author. Fax: (1)-412-383 2123.
E-mail address: kagan@pitt.edu (V.E. Kagan).

Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-MCA; AMC, 7-amino-4-methyl-coumarin; APT, aminophospholipid translocase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, propidium iodide; PnA, *cis*-parinaric acid; PS, phosphatidylserine; ROS, reactive oxygen species; STS, staurosporine

0014-5793/02/$22.00
PII: S0014-5793(02)02990-3

Obviously, a model of non-oxidant-induced apoptosis should be evaluated to avoid the confounding effects of non-specific oxidation.

Therefore, we used a model of staurosporine (STS)-induced apoptosis to determine whether PS oxidation may be recognized as a selective oxidative signal associated with the execution of apoptotic program. It has been shown that STS, a potent protein kinase C inhibitor with a broad spectrum of activity [16], induces apoptotic cell death in a number of different cell lines [4,17–20]. Importantly, STS also induces oxidative stress in various cells even though it does not act as a chemical oxidant [4,21–23].

In the present study, we assessed PS oxidation and externalization during STS-induced apoptosis in HL-60 cells. We demonstrated that PS undergoes pronounced oxidation as compared to PC, the most abundant membrane phospholipid, during STS-induced apoptosis. We further showed that this PS oxidation occurred concurrently with the onset of (1) cytochrome *c* release, (2) PS externalization and (3) phagocytosis of the apoptotic cells by macrophages. These results strongly suggest that generation of ROS and subsequent PS oxidation are features of early apoptosis and likely participate in PS-dependent signaling pathways involved in clearance of apoptotic cells.

2. Materials and methods

2.1. Materials

STS, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), reduced glutathione (GSH) and Hoechst 33342 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC solvents (methanol, chloroform, and hexane) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 7-Amino-4-methyl-coumarin (AMC) and acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-AMC) were purchased from Peptides International (Louisville, KY, USA). ThioGlo-1™ maleimide reagent was obtained from Covalent Associates Inc. (Woburn, MA, USA). Cell Tracker Orange™ and PnA (Z-9, E-11, E-13, Z-15-octadecatetraenoic acid) were purchased from Molecular Probes (Eugene, OR, USA). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were obtained from Gibco BRL Products (Grand Island, NY, USA). The purity of PnA was determined by UV spectrophotometry at 304 nm in ethanol ($\varepsilon = 80$ mM^{-1} cm^{-1}). All other chemicals were of analytical grade.

2.2. Cell culture

Human promyelocytic leukemia HL-60 cells were grown in RPMI 1640 medium supplemented with 12.5% heat-inactivated FBS at 37°C in a humidified incubator and 5% CO_2+95% air. Cells were seeded at a concentration of 2×10^5 cells/ml and logarithmic growth was maintained by passaging every 2–3 days. All current experiments were performed using RPMI 1640 medium containing 12.5% FBS and 1×10^6 cells/ml, unless specified otherwise.

Murine macrophages (J774A.1, cell line obtained from ATCC) were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin sulfate and were incubated in a humidified atmosphere (5% CO_2+95% air) at 37°C.

2.3. Determination of apoptotic cells by nuclear morphology

HL-60 cells (1×10^6 cells/ml) were incubated with 1 μM STS at 37°C for 1–6 h, washed with phosphate-buffered saline (PBS), and then fixed with 2% glutaraldehyde for 2 h. Samples (1×10^6 cells/100 μl) were then centrifuged, washed, resuspended with PBS, and stained with Hoechst 33342 (5 μg/ml). They were subsequently mounted on glass slides and observed under a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with digital Hamamatsu CCD camera (C4742-95-12NBR). Results were expressed as the percentage of the cells showing characteristic nuclear morphological features of apoptosis (nuclear condensation and fragmentation) relative to the total number of counted cells. (For each time point, ≥ 200 total cells were evaluated.)

2.4. Determination of caspase-3 activity

The activity of caspase-3 was determined as described previously [24]. Briefly, after STS treatment, cells were collected, washed in PBS, and lysed for 20 min on ice in lysis buffer containing 10 mM HEPES–KOH (pH 7.4); 2 mM EDTA; 0.1% CHAPS; 1 mM phenylmethylsulfonyl fluoride (PMSF); and 5 mM dithiothreitol (DTT). The suspensions were centrifuged at 4°C and the supernatants were collected as lysates. For measurement of caspase activity, 10 μg of lysate was diluted to 20 μl with lysis buffer, mixed with 20 μl $2\times$ICE buffer (40 mM HEPES–KOH (pH 7.4), 20% (v/v) glycerol, 1 mM PMSF and 4 mM DTT) containing 40 μM Ac-DEVD-AMC (a fluorogenic peptide substrate), and incubated for 60 min at 37°C. After 60 min, 460 μl of distilled water was added, and the fluorescence was measured in a CytoFluor 2350 (Millipore, Bedford, MA, USA) fluorescence microplate reader set at an excitation wavelength of 360 ± 40 nm and an emission of 460 ± 40 nm. One unit of caspase activity was defined as the amount of enzyme required to release 1 pmol AMC per min.

The protein concentration of cell lysates was measured by the method of Bradford [25].

2.5. Flow cytometry of PS externalization

Annexin V binding to cells was determined using a commercially available staining kit (Biovision, Mountain View, CA, USA) and flow cytometry as previously described [26]. Briefly, after treatment with STS, HL-60 cells were recovered and washed once with PBS. Cells were labeled with FITC-conjugated annexin V and propidium iodide (PI) for 5 min, and immediately analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) with simultaneous monitoring of green fluorescence (530 nm, 30 nm band-pass filter) for annexin V–FITC and red fluorescence (long-pass emission filter that transmits light >650 nm) associated with PI.

2.6. Determination of phospholipid peroxidation in HL-60 cells

PnA is a natural 18-carbon fatty acid with four conjugated double bonds. PnA is highly susceptible to peroxidation and also fluorescent. The fluorescence is irreversibly lost upon peroxidation, providing a convenient approach for quantifying lipid peroxidation [27,28]. PnA is used as a reporter molecule for assay of membrane lipid peroxidation in intact mammalian cells. PnA was metabolically incorporated into HL-60 cell phospholipids (1×10^6 cells/ml) by addition of PnA–human serum albumin complex to give a final concentration of 2.5 μg PnA/10^6 cells in FBS-free RPMI 1640 medium without phenol red. Cells were incubated for 2 h at 37°C in the dark. Thereafter, PnA-labeled cells were resuspended in RPMI 1640 supplemented with 12.5% FBS and incubated with 1 μM STS at 37°C for an indicated period of time. At the end of each incubation period, phospholipid oxidation was determined according to previously described methods [11].

2.7. Preparation of mitochondrial and cytosolic fractions and quantification of cytochrome c

HL-60 cells were treated with 1 μM STS, harvested, and washed three times with ice-cold PBS. Cells were resuspended in isotonic buffer A (10 mM HEPES, 0.3 M mannitol and 0.1% BSA), supplemented with 0.1 mM digitonin (1.5×10^6 cells/ml), left on ice for 5 min, and immediately centrifuged at $8500\times g$ for 5 min at 4°C. The collected supernatant was used as the cytosolic fraction. The pellet was resuspended in sonication buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF and 0.5% Tween 20), sonicated three times (20 s each) on ice, and centrifuged at $10\,000\times g$ for 30 min at 4°C; the resulting supernatant was used as the mitochondrial fraction. The amount of cytochrome *c* in mitochondrial and cytosolic fractions was measured with a commercially available cytochrome *c* ELISA kit (Oncogene Research Products, San Diego, CA, USA) according to the manufacturer's instructions, after appropriate dilution.

2.8. Determination of intracellular GSH contents

HL-60 cells (1×10^6 cells/ml) were incubated (at 37°C in 5% CO_2) in the absence or presence of 1 μM STS in RPMI 1640 medium for 0, 1, 2, 4, and 6 h. After treatments, cells were harvested, and the glutathione content was determined fluorometrically using ThioGlo-1™ as previously described [29]. Briefly, harvested treated and untreated cells

were washed and resuspended with PBS and lysed by freezing and thawing once. Immediately after adding 10 μM ThioGlo-1™ to the lysates, fluorescence was measured in a CytoFluor 2350 (Millipore) fluorescence microplate reader set at an excitation wavelength of 360 ± 40 nm and emission, at 530 ± 25 nm.

The protein concentration of cell lysates was measured by the method of Bradford [25].

2.9. Phagocytosis of HL-60 cells by macrophages

Macrophage J774A.1 cells were used for phagocytosis assays. Before adding target (STS-treated or untreated HL-60) cells, macrophages were seeded into an 8-well chamber slide (5×10^4 cells/well) and were cultured overnight. HL-60 cells were labeled with Cell Tracker Orange™ for 15 min at 37°C and then exposed to 1 μM STS for 3 h. The cells were subsequently washed and resuspended in serum-containing RPMI medium. Fluorescently labeled cells (5×10^5 cells/well) were added to macrophage cultures, and each mixture was incubated for 1 h at 37°C; unbound cells were then washed three times with RPMI medium and then three times with PBS. Well contents were fixed and stained with a fixing solution (2% paraformaldehyde, in PBS containing 1 μg/ml Hoechst 33342 (a blue fluorescent stain for nuclei)) for 30 min at room temperature. The cells were examined under a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with digital Hamamatsu CCD camera (C4742-95-12NBR) and analyzed using the MetaImaging Series™ software v.4.6 (Universal Imaging Corp., Downingtown, PA, USA). A minimum of 500 macrophages were analyzed per experimental condition. Results were expressed as the percentage of the phagocytosis-positive macrophages.

2.10. Statistical evaluations

Data are expressed as mean ± standard error of the mean (S.E.M.). Changes in variables for different assays were analyzed either by Student's *t*-test (single comparisons) or by one-way ANOVA for multiple comparisons. Differences were considered to be significant at $P<0.05$.

3. Results

3.1. Time-course of STS-induced apoptosis in HL-60 cells

To characterize apoptosis in HL-60 cells, we studied the time-course (from 1 to 6 h) of (i) the appearance of typical nuclear morphology in the cells, (ii) caspase-3 activation, (iii) PS externalization, and (iv) release of cytochrome *c* from mitochondria after the cells were exposed to STS.

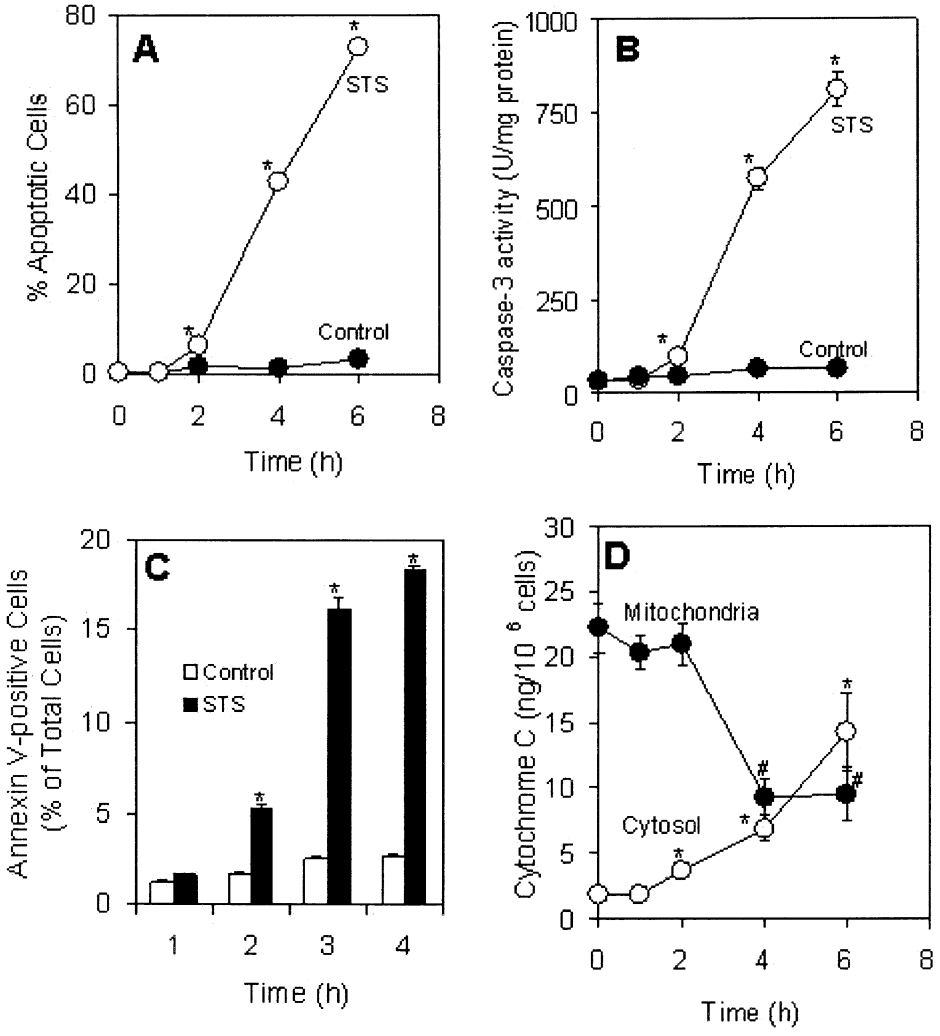

Fig. 1. Time-course of biomarkers of apoptosis in HL-60 cells exposed to STS. Cells (1×10^6 cells/ml) were incubated at 37°C under 5% CO_2/95% air atmosphere in the absence or presence of 1 μM STS for the indicated time periods. A: Apoptotic cells were determined by nuclear morphological examination with Hoechst 33342 staining. B: Caspase-3 activity was measured using fluorescent substrates. Data points represent the mean ± S.E.M. of three to five separate experiments. Note: some error bars are too small to be seen. (*$P<0.01$ vs. control at the same time period). C: PS externalization was determined by annexin V binding assayed by flow. D: Cytochrome *c* content in the cytosolic (open circles) and mitochondrial (solid circles) fractions assayed by ELISA. Data are mean ± S.E.M. of three separate experiments. *$P<0.01$ vs. 0-h control for cytosol, #$P<0.01$ vs. 0-h control for mitochondria.

3.1.1. Accumulation of apoptotic cells. Apoptotic cells became evident 2 h after STS treatment, as assessed by examination of nuclear morphology; the number of apoptotic cells increased with time up to 6 h (Fig. 1A) in a manner similar to caspase-3 activation (Fig. 1B). In control cell cultures, no accumulation of apoptotic cells was observed.

3.1.2. Caspase-3 activation. After treatment of cells with STS, a slight but significant increase in caspase-3 activity was observed at 2 h; the activity increased prominently and in a time-dependent manner at later time points (Fig. 1B). Caspase-3 activation was not detected in control cells.

3.1.3. PS externalization. PS externalization from the inner leaflet of the plasma membrane to its outer leaflet is known to be one of early markers of apoptosis [14]. A significant accumulation of apoptotic HL-60 cells with externalized PS (defined as annexin V-positive and PI-negative cells) commenced 2 h after treatment with STS and increased in a time-dependent manner (Fig. 1C). Notably, the amounts of annexin V-positive/PI-positive (late apoptotic+necrotic) cells during STS-induced apoptosis were relatively low and did not exceed 5.5% of the total number of cells (data not shown).

3.1.4. Cytochrome c efflux from mitochondria into the cytosol. At 2, 3 and 4 h after STS treatment, the contents of cytochrome *c* in the cytosol increased 2-, 4-, and 8-fold, respectively, when compared to that of the controls (time point 0). Conversely, *mitochondrial* cytochrome *c* levels decreased after exposure of cells to STS although statistical significance was reached only after 3 h (Fig. 1D).

3.2. Time-course of STS-induced oxidative stress in HL-60 cells

Two end-points were assessed to characterize STS-induced oxidative stress in HL-60 cells: (i) peroxidation of membrane phospholipids and (ii) loss of intracellular GSH.

3.2.1. Phospholipid peroxidation. Recall that metabolic labeling of phospholipids with oxidation-sensitive fluorescent PnA and HPLC with fluorescence detection was used to assay phospholipid peroxidation [11]. We found that the most abundant membrane phospholipid in HL-60 cells, PC, *did not* undergo statistically significant peroxidation after 0.5, 1.0, or 2.0 h of STS treatment (Fig. 2A). In contrast, PS showed a statistically significant peroxidation (detected as a loss of fluorescent PnA–PS) relative to that of the control cells after 2 h exposure to STS (Fig. 2A). No significant PS oxidation was detected at earlier time points (0.5 and 1.0 h). Unfortunately, intrinsic fluorescence of STS precluded analysis of peroxidation levels of PE and phosphatidylinositol because fluorescent STS co-eluted with this phospholipid, interfering with accurate detection of its respective peak.

3.2.2. GSH depletion. Measurements of GSH contents in STS-treated HL-60 cells revealed an approximately 25% decrease in GSH concentration occurring only 4 h after STS treatment. At 6 h after STS exposure, the cells lost $\approx 50\%$ of their GSH (Fig. 2B). No significant changes in GSH content were detectable during the initial 2 h of STS exposure.

3.3. Phagocytosis of STS-treated HL-60 cells by J774A.1 macrophages

Externalization of PS on the surface of plasma membrane acts as a distinctive signal that allows macrophages to recognize and phagocytize apoptotic cells [15]. Because STS-induced apoptosis was accompanied by PS externalization, we next determined whether recognition and uptake of HL-60 apoptotic cells by macrophages takes place. The percentage of phagocytosis-positive J774A.1 macrophages with bound and/or phagocytized STS-treated (1 μM, 3 h) HL-60 cells was approximately $13 \pm 1\%$ – a significant increase when compared with $4 \pm 1\%$ phagocytosis-positive macrophages observed after co-incubation with untreated HL-60 cells.

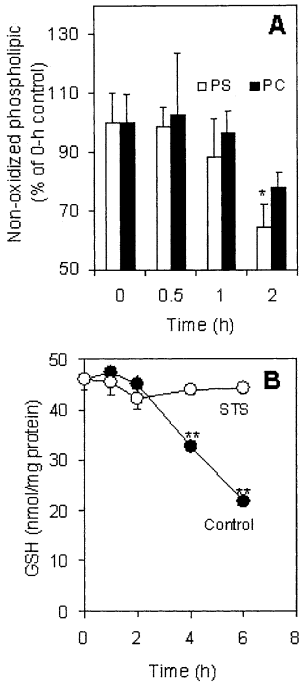

Fig. 2. Time-course of biomarkers of oxidative stress in HL-60 cells treated with STS. A: Phospholipid peroxidation assayed by fluorescence HPLC of PnA-labeled phospholipids. PnA phospholipids (PS, filled columns and PC, clear columns) were detected fluorometrically at 420 nm under excitation at 324 nm. Data points represent the mean ± S.E.M. of three to five separate experiments ($^*P < 0.05$ vs. 0-h control). B: Intracellular GSH determined fluorometrically using ThioGlo-1™. Data points represent the mean ± S.E.M. of three to four separate experiments ($^{**}P < 0.01$ vs. control at the same time period). Note: some error bars are too small to be seen.

4. Discussion

Oxidative stress is both an inducer of apoptosis and an inherent part of the apoptosis execution program (reviewed in [1]). H_2O_2 [7,30,31], peroxynitrite [32,33], and nitric oxide generators [34,35] are known to induce apoptosis in a variety of cell types. Furthermore, inhibition of apoptosis by different antioxidants such as *N*-acetyl cysteine [36–38], pyrrolidine dithiocarbamate [39], ascorbate [40], and α-tocopherol [40], and antioxidant enzymes such as catalase [36] suggest that generation of ROS plays a role in apoptosis in diverse cell lines [38,41]. However, no specific function for ROS or oxidation products in apoptotic signaling pathways has been identified.

We have previously demonstrated that involvement of PS in peroxidation during oxidant-induced apoptosis in different cell lines was disproportionally greater than that of other more abundant phospholipids such as PC and PE [17]. Given that transmembrane migration and subsequent externalization of PS is an important signaling pathway for recognition of apoptotic cells by macrophages, we hypothesized that PS oxidation in plasma membrane may play some, as yet unrecog-

nized, role in its externalization. We further speculated that preferential oxidation of negatively charged PS during apoptosis may be related to its binding of positively charged cytochrome *c*, which, once released from mitochondria, catalyzes PS oxidation [13]. Experimental testing of this hypothesis in oxidant-induced models of oxidative stress is difficult, if not impossible, because of the high level of non-specific background phospholipid peroxidation generated by the oxidants. This can, however, be determined in models of non-oxidant-induced apoptosis, during which only specific phospholipid peroxidation associated with execution of the apoptotic program is likely to occur.

In the present work, we chose to use STS to induce apoptosis and associated oxidative stress in HL-60 cells. This is because STS – a non-specific inhibitor of protein kinases [16] as well as a potent inducer of apoptosis [4,17–20] – has been reported to induce PS externalization [18,42], cytochrome *c* release from mitochondria into the cytosol [4,5,17,19,20,22,42], and ROS generation [4,21–23] during apoptosis. We found that, indeed, STS was very effective in inducing apoptosis in HL-60 cells as evidenced by characteristic changes of nuclear morphology, caspase-3 activation, and, importantly, PS externalization. Essentially, the time-courses of all these biomarkers of apoptosis revealed a common pattern: a slight but significant elevation 2 h after STS exposure of HL-60 cells, with a dramatic increase at later time points.

In contrast, our assessment of oxidative stress did not show a uniform response to STS. The most abundant intracellular oxidation substrate, water-soluble antioxidant GSH, did not show any significant oxidation during a 2-h period after treatment of cells with STS and only underwent oxidation after 4 h of STS exposure; similarly, oxidation of the most abundant membrane phospholipid, PC, was not detectable for 0.5–2.0 h of STS exposure. Remarkably, a pronounced peroxidation of PS was detected after a 2-h exposure to STS, although it was not detectable at earlier time points (0.5 and 1.0 h). This preferential oxidation of PS (as compared to more abundant PC) at early stages of STS-induced apoptosis raises questions about the potential catalytic mechanisms involved in this process.

We have previously hypothesized that release of cytochrome *c* from mitochondria into the cytosol may facilitate its electrostatic interactions with PS, resulting in the oxidation of the latter [13]. This catalytic process requires that ROS (O_2^- and H_2O_2) be produced in excess by disrupted mitochondrial electron transport [4,5]. In the present study, we found that relatively small but significantly increased amounts of cytochrome *c* were detectable in the cytosol of HL-60 cells 2 h after STS exposure – at the time when PS oxidation was observed. Although cytochrome *c* may act as a superoxide scavenger and an antioxidant during electron transport in mitochondria [43,44] it is likely that it functions as a catalytic heme-containing prooxidant after being released along with hydrogen peroxide into the cytosol during STS-induced apoptosis. In fact, our results suggest that relatively small amounts of cytochrome *c* in the cytosol are quite sufficient for catalytic PS peroxidation, although similarity of the time-course for cytochrome *c* release and PS oxidation may be an occasional coincidence. Notably, these low concentrations of cytochrome *c* in the cytosol are inadequate for GSH or PC oxidation, implying that specific mechanisms may be involved in PS oxidation. The actual catalytic mechanisms underlying PS oxidation during apoptosis and the specific role of cytochrome *c* in the catalysis remain to be elucidated.

Phospholipid transbilayer asymmetry is primarily maintained by two enzymes in plasma membrane [45]: one is an inward-directed pump – an ATP-dependent transporter specific for aminophospholipids known as aminophospholipid translocase (APT); the other is a phospholipid scramblase that facilitates bi-directional migration of all phospholipid classes. PS externalization during apoptosis is thought to result from an inhibition of APT activity combined with the activation of scramblase [45,46]. Although it is still unclear whether PS oxidation can drive exposure of PS on the outer leaflet, some potential mechanisms have been proposed. Since APT is sensitive to oxidative stress [47], ROS and/or oxidized PS might inhibit APT activity directly. Alternatively, oxidized PS (in contrast to non-oxidized PS) might avoid APT-dependent transport because of its poor recognition by the enzyme or because of its rapid flip-flop movement across the membrane [48]. Further studies are required to reveal the exact relationships between PS oxidation and externalization. Nevertheless, our results indicate that both pronounced PS oxidation and externalization occur in STS-exposed HL-60 cells, rendering them a target for phagocytosis. Indeed, treatment of cells with STS yielded a substantial enhancement of their binding and uptake by J774A.1 macrophages.

Overall, we established that although STS does not induce apoptosis by way of non-specific oxidative stress, execution of the STS-induced apoptotic program is accompanied by an early pronounced oxidation of PS. The results of our time-course studies are compatible with the conclusion that PS oxidation (i) involves PS interaction with cytochrome *c* released into the cytosol, where the latter utilizes H_2O_2 and other ROS for selective oxidative catalysis; (ii) facilitates PS externalization on the outer surface of plasma membrane; and (iii) serves as an additional recognition signal that enhances phagocytosis of apoptotic cells.

Acknowledgements: Supported by grants from NIH 1RO1HL70755-01 and HL64145-01A1. The authors thank Dr. Teresa L. Bradley for her valuable help in preparing the manuscript.

References

[1] Buttke, T.M. and Sandstrom, P.A. (1994) Immunol. Today 15, 7–10.
[2] Jabs, T. (1999) Biochem. Pharmacol. 57, 231–245.
[3] Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.A., Petit, P.X., Mignotte, B. and Kroemer, G. (1995) J. Exp. Med. 182, 367–377.
[4] Cai, J. and Jones, D.P. (1998) J. Biol. Chem. 273, 11401–11404.
[5] Bossy-Wetzel, E., Newmeyer, D.D. and Green, D.R. (1998) EMBO J. 17, 7–49.
[6] Crompton, M. (1999) Biochem. J. 341, 233–249.
[7] Hammpton, M.B. and Orrenius, S. (1997) FEBS Lett. 414, 552–556.
[8] Marks, F., Muller-Decker, K. and Furstenberger, G. (2000) Toxicology 153, 11–26.
[9] Serhan, C.N. (1997) Prostaglandins 53, 107–137.
[10] Roberts II, L.J., Salomon, R.G., Morrow, J.D. and Brame, C.J. (1999) FASEB J. 13, 1157–1168.
[11] Ritov, V.B., Banni, S., Yalowich, J.C., Day, B.W., Claycamp, H.G., Corongiu, F.P. and Kagan, V.E. (1996) Biochim. Biophys. Acta 1283, 127–140.
[12] Tyurina, Y.Y., Shvedova, A.A., Kawai, K., Tyurin, V.A., Kommineni, C., Quinn, P.J., Schor, N.F., Fabisiak, J.P. and Kagan, V.E. (2000) Toxicology 148, 93–101.

[13] Kagan, V.E., Fabisiak, J.P., Shvedova, A.A., Tyurina, Y.Y., Tyurin, V.A., Schor, N.F. and Kawai, K. (2000) FEBS Lett. 477, 1–7.
[14] Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M. and Green, D.R. (1995) J. Exp. Med. 182, 1545–1556.
[15] Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L. and Henson, P.M. (1992) J. Immunol. 148, 2207–2216.
[16] Ruegg, U.T. and Burgess, G.M. (1989) Trends Pharmacol. Sci. 10, 218–220.
[17] Wolf, C.M. and Eastman, A. (1999) Exp. Cell Res. 247, 505–513.
[18] Belmokhtar, C.A., Hillion, J. and Segal-Bendirdjian, E. (2001) Oncogene 20, 3354–3362.
[19] Yuste, V.J., Sanchez-Lopez, I., Sole, C., Encinas, M., Bayascas, J.R., Boix, J. and Comella, J.X. (2002) J. Neurochem. 80, 126–139.
[20] Paul, C., Manero, F., Gonin, S., Kretz-Remy, C., Virot, S. and Arrigo, A.P. (2002) Mol. Cell. Biol. 22, 816–834.
[21] Kruman, I., Guo, Q. and Mattson, M.P. (1998) J. Neurosci. Res. 51, 293–308.
[22] Pong, K., Doctrow, S.R., Huffman, K., Adinolfi, C.A. and Baudry, M. (2001) Exp. Neurol. 171, 84–97.
[23] Ahlemeyer, B., Bauerbach, E., Plath, M., Steuber, M., Heers, C., Tegtmeier, F. and Krieglstein, J. (2001) Free Radic. Biol. Med. 30, 1067–1077.
[24] Kagaya, S., Kitanaka, C., Noguchi, K., Mochizuki, T., Sugiyama, A., Asai, A., Yasuhara, N., Eguchi, Y., Tsujimoto, Y. and Kuchino, Y. (1997) Mol. Cell. Biol. 17, 6736–6745.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Fabisiak, J.P., Tyurina, Y.Y., Tyurin, V.A., Lazo, J.S. and Kagan, V.E. (1998) Biochemistry 37, 13781–13790.
[27] Sklar, L.A., Hudson, B.S., Petersen, M. and Diamond, J. (1997) Biochemistry 16, 813–819.
[28] Kuypers, F.A., van den Berg, J.J., Schalkwijik, C., Roelofsen, B. and Op den Kamp, J.A. (1987) Biochim. Biophys. Acta 921, 266–274.
[29] Kagan, V.E., Yalowich, J.C., Borisenko, G.G., Tyurina, Y.Y., Tyurin, V.A., Thampatty, P. and Fabisiak, J.P. (1999) Mol. Pharmacol. 56, 494–506.
[30] Matsura, T., Kai, M., Fujii, Y., Ito, H. and Yamada, K. (1999) Free Radic. Res. 30, 73–83.
[31] Ueda, N. and Shah, S.V. (1992) J. Clin. Invest. 90, 2593–2597.
[32] Lin, K.T., Xue, J.Y., Normen, M., Spur, B. and Wong, P.Y.K. (1995) J. Biol. Chem. 270, 16487–16490.
[33] Salgo, M.G. and Pryor, W.A. (1996) Arch. Biochem. Biophys. 333, 482–488.
[34] Yabuki, M., Kariya, S., Inai, Y., Hamazaki, K., Yoshioka, T., Yasuda, T., Horton, A.A. and Utsumi, K. (1997) Free Radic. Biol. Med. 27, 325–335.
[35] Brockhaus, F. and Brune, B. (1998) Exp. Cell Res. 238, 33–41.
[36] Luo, Y., Umegaki, H., Wang, X., Abe, R. and Roth, G.S. (1998) J. Biol. Chem. 273, 3756–3764.
[37] Um, H.D., Orenstein, J.M. and Wahl, S.M. (1996) J. Immunol. 56, 3469–3477.
[38] Lizard, G., Gueldry, S., Sordet, O., Monier, S., Athias, A., Miguet, C., Bessede, G., Lemaire, S., Solary, E. and Gambert, P. (1998) FASEB J. 12, 1651–1663.
[39] Bessho, R., Matsubara, K., Kubota, M., Kuwakado, K., Hirota, H., Wakazono, Y., Lin, Y.W., Okuda, A., Kawai, M., Nishimori, R. and Heike, T. (1994) Biochem. Pharmacol. 48, 1883–1889.
[40] Barroso, M.P., Gomez-Diaz, C., Lopez-Lluch, G., Malagon, M.M., Crane, F.L. and Navas, P. (1997) Biochem. Biophys. 343, 243–248.
[41] Valencia, A. and Moran, J. (2001) J. Neurosci. Res. 64, 284–297.
[42] Denecker, G., Dooms, H., van Loo, G., Vercammen, D., Grooten, J., Fiers, W., Declercq, W. and Vandenabeele, P. (2000) FEBS Lett. 465, 47–52.
[43] Skulachev, V.P. (1998) FEBS Lett. 423, 275–280.
[44] Korshunov, S.S., Krasnikov, B.F., Pereverzev, M.O. and Skulachev, V.P. (1999) FEBS Lett. 462, 192–198.
[45] Zwaal, R.F. and Schroit, A.J. (1997) Blood 89, 1121–1132.
[46] Fadok, V.A., Bratton, D.L., Frasch, S.C., Warner, M.L. and Henson, P.M. (1998) Cell Death Differ. 5, 551–562.
[47] Herrmann, A. and Devaux, P.F. (1990) Biochim. Biophys. Acta 1027, 41–46.
[48] Barsukov, L.I., Victorov, A.V., Vasilenko, I.A., Evstingeeva, R.P. and Bergelson, L.D. (1980) Biochim. Biophys. Acta 598, 153–168.