NADPH Oxidase-dependent Oxidation and Externalization of Phosphatidylserine during Apoptosis in Me₂SO-differentiated HL-60 Cells

ROLE IN PHAGOCYTIC CLEARANCE*

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Resolution of inflammation requires clearance of activated neutrophils by phagocytes in a manner that protects adjacent tissues from injury. Mechanisms governing apoptosis and clearance of activated neutrophils from inflamed areas are still poorly understood. We used dimethylsulfoxide-differentiated HL-60 cells showing inducible oxidase activity to study NADPH oxidase-induced apoptosis pathways typical of neutrophils. Activation of the NADPH oxidase by phorbol myristate acetate caused oxidative stress as shown by production of superoxide and hydrogen peroxide, depletion of intracellular glutathione, and peroxidation of all three major classes of membrane phospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. In addition, phorbol myristate acetate stimulation of the NADPH oxidase caused apoptosis, as evidenced by apoptosis-specific phosphatidylserine externalization, increased caspase-3 activity, chromatin condensation, and nuclear fragmentation. Furthermore, phorbol myristate acetate stimulation of the NADPH oxidase induced recognition and ingestion of dimethylsulfoxide-differentiated HL-60 cells by J774A.1 macrophages. To reveal the apoptosis-related component of oxidative stress in the phorbol myristate acetate-induced response, we pretreated cells with a pancaspase inhibitor, benzoyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), and found that it caused partial inhibition of hydrogen peroxide formation as well as selective protection of only phosphatidylserine, whereas more abundant phospholipids, phosphatidylcholine and phosphatidylethanolamine, were oxidized to the same extent in the absence or presence of z-VAD-fmk. In contrast, inhibitors of NADPH oxidase activity, diphenylene iodonium and staurosporine, as well as antioxidant enzymes, superoxide dismutase/catalase, completely protected all phospholipids against peroxidation, inhibited expression of apoptotic biomarkers and externalization of phosphatidylserine, and reduced phagocytosis of differentiated HL-60 cells by J774A.1 macrophages. Similarly, zymosan-induced activation of the NADPH oxidase resulted in the production of superoxide and oxidation of different classes of phospholipids of which only phosphatidylserine was protected by z-VAD-fmk. Accordingly, zymosan caused apoptosis in differentiated HL-60 cells, as evidenced by caspase-3 activation and phosphatidylserine externalization. Finally, zymosan triggered caspase-3 activation and extensive SOD/catalase-inhibitable phosphatidylserine exposure in human neutrophils. Overall, our results indicate that NADPH oxidase-induced oxidative stress in neutrophil-like cells triggers apoptosis and subsequent recognition and removal of these cells through pathways dependent on oxidation and externalization of phosphatidylserine.

Neutrophils aid host defense by killing invading microorganisms through production of highly reactive oxygen species (ROS)1 generated by activation of the NADPH oxidase complex. When released inappropriately into the extracellular milieu, these ROS can cause persistent inflammation and considerable damage to the surrounding, healthy tissues. To prevent calamitous release of ROS, macrophages remove excess activated neutrophils from an inflammatory site in a regulated way, through processes that ensure swift resolution of inflammation yet make provision for neutrophils to fulfill their microbicidal function. Phagocytic cells carry out this clearance by recogniz-

* This work was supported by National Institutes of Health Grants 1R01HL64145-01A1 and 1R01HL70755-01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ Fellow of Spanish Ministry of Education and Cultura Grant PB98-0329-CO2-02.

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Valerian E. Kagan is a Fellow of the Spanish Ministerio de Educació n y Cultura and supported by the Spanish Ministry of Education, Cultura y Deportes (Secretaría de Estado de Educación y Universidades).

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The abbreviations used are: ROS, reactive oxygen species; z-VAD-fmk, benzoyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone; DPI, diphenylene iodonium; FBS, fetal bovine serum; MCLA, 2-methyl-6-(4-thiobenzyloxy)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride; MPO, myeloperoxidase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMA, phorbol 12-myristate 13-acetate; PnA, cis-parinaric acid; PS, phosphatidylserine; SOD, superoxide dismutase; HFLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; CHAPS, 3-[(3-cholamidopropyl)dimethyammonio]-1-propanesulfonic acid.
ing apoptotic neutrophils through a mechanism that involves the exposure of phosphatidylserine (PS) on the neutrophil cell surface (1–6).

Neutrophils are short lived; in the absence of inflammation, resting neutrophils undergo apoptosis in the circulation after 6–9 h (7). Conversely, when neutrophils reach a site of inflammation, apoptosis is delayed by inflammatory cytokines in the tissues, providing additional time for completion of the neutrophil’s microbicidal function (8). However, neutrophils also become activated when they migrate from the blood; their subsequent production of ROS generates oxidative stress that instigates apoptosis (9, 10). Redox-dependent mechanisms for apoptosis include the intracellular production of superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) by activation of the NADPH oxidase in the granule pool (9) or the down-regulation of key antioxidant systems of the cell, such as superoxide dismutase (SOD), and a decrease in GSH (10). In addition, macrophages and phagocytic cells in the inflammatory area release Fas ligand during phagocytosis, contributing further to triggering apoptosis in neutrophils (11). Altogether, the organism maintains a delicate balance among ROS production, cytokine-induced delay of apoptosis, and ROS-promoted neutrophilic death to protect the host tissues from excessive oxidative injury.

Although the involvement of the NADPH oxidase in neutrophil apoptosis has been demonstrated (9, 10), specific signaling pathways through which oxidative stress participates in recognition and clearance of apoptotic neutrophils have not been elucidated. We have previously shown that specific oxidation and externalization of PS was characteristic of oxidant-induced apoptosis in several different cell lines (12). We further hypothesized that NADPH oxidase-induced oxidative stress plays a specific role in recognition and clearance, a role realized through selective oxidation of PS, associated with PS externalization on the neutrophilic cell surface, and subsequent recognition of apoptotic cells by macrophages. In the present work, we used Me$_2$SO-differentiated HL-60 cells as a model to study NADPH oxidase-induced apoptosis pathways typical of neutrophils. In a separate series of experiments, we also used human neutrophils to corroborate our findings.

Me$_2$SO-differentiated HL-60 cells, in contrast to their nondifferentiated parental cells, possess a complete NADPH oxidase system (13) that can be activated by various agents (phorbol esters, chemoattractant peptides, and phagocytosable particles such as opsonized zymosan, calcium ionophores, etc.). Our results suggest the following sequence of events in the apoptotic execution program of Me$_2$SO-differentiated HL-60 cells stimulated with phorbol 12-myristate 13-acetate (PMA) or opsonized zymosan: (i) activation of NADPH oxidase-dependent O$_2^-$ and H$_2$O$_2$ production; (ii) oxidation of different classes of phospholipids of which only PS was specifically protected by a pancaspase inhibitor, z-VAD-fmk; (iii) externalization of PS on the cell surface; and (iv) recognition and clearance of apoptotic cells by the macrophage cell line J774A.1 (after PMA stimulation). The involvement of the NADPH oxidase in this signaling cascade has been evidenced by the inhibitory effect of diphenylethyl iodonium (DPI) and staurosporine as well as by a combination of SOD/catalase. Thus, we present evidence of a new signaling pathway for apoptosis in which oxidative stress is inherently involved in PS oxidation and externalization and its subsequent recognition and through which neutrophilic cells facilitate their own clearance by macrophages.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

Fetal bovine serum (FBS), Me$_2$SO, PMA, SOD, catalase, DPI, staurosporine, cytochrome c from horse heart, GSH, Hoescht 33342, protamine K, Trias-acetate-EDTA buffer, RNase A, 3-amino-1,2,4-triazole, guanosine, fluorescamine, and zymosan were purchased from Sigma. HPLC solvents (methanol, chloroform, hexane, and water) were obtained from Aldrich. Cetyltrimethylammonium bromide was purchased from Acros Organics (Pittsburgh, PA). 7-Amino-4-methylcoumarin and acetyl-Asp-Glu-Val-Asp-4-methylcoumarin were purchased from Peptides International (Louisville, KY). ThioGlo-1™ maleimide reagent was from Covalent Associates Inc. (Woburn, MA). 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol(1,2α)-pyrazin-3-one, hydrochloride (MCLA); Amprex Red hydrogen peroxide assay kit; and cis-parinaric acid (PnA) (Z-9, E-11, E-13, Z-15-octadecatetraenoic acid) dihydro- rhodamine 123 were purchased from Molecular Probes Inc. (Eugene, OR). Pancaspase inhibitor, z-VAD-fmk were purchased from Calbiochem, La Jolla, CA. Dulbecco’s modified Eagle’s medium and RPMI 1640 medium, agarose, and 100-bp DNA ladder standards were obtained from Invitrogen Inc. (Carlsbad, CA). The purity of PnA was determined by UV spectrophotometry at 304 nm in ethanol (ε = 80 mm$^{-1}$ cm$^{-1}$). All other chemicals used were of analytical grade.

**Cell Cultures and Treatments**

HL-60 human promyelocytic leukemia cells were maintained in RPMI 1640 medium supplemented with 12.5% heat inactivated FBS at 37 °C and in a humidified atmosphere (5% CO$_2$ plus 95% air). Cells were seeded at a density of 5 x 10$^6$ ml$^{-1}$ and grown for 6 days in the presence of 1.25% Me$_2$SO to induce differentiation to the neutrophilic lineage. Fresh medium with Me$_2$SO was added on the third day of culture to prevent cell overgrowth and depletion of nutrients (14). Several different criteria were utilized to characterize the effectiveness of the differentiation process (15): (i) an increase of NADPH oxidase activity from <0.05 in nondifferentiated HL-60 cells to 1.25 nmol of superoxide/min/10$^6$ cells in Me$_2$SO-treated PMA-stimulated HL-60 cells; (ii) a 10-fold decrease of MPO activity (from 55.25 ± 9.16 to 4.62 ± 1.30 nmol of guaiacol reduced/min/10$^6$ cells before and after Me$_2$SO treatment, respectively); (iii) the fact that over 95% of Me$_2$SO-treated cells were viable and had a significantly smaller size than parental nondifferentiated HL-60 cells (14); and (iv) FACScan analysis to determine the number of Me$_2$SO-differentiated HL-60 cells after stimulation with PMA using dihydro- rhodamine 123 staining for the NADPH oxidase. After PMA stimulation, 97% ± 1% of Me$_2$SO-differentiated HL-60 cells were responsive to dihydro- rhodamine 123 (5 μg/ml) staining, whereas less than 1% of nondifferentiated cells appeared to be dihydro- rhodamine 123-positive (16). After 6 days, cells were collected by centrifugation at 1000 × g for 5 min, washed in prewarmed PBS buffer (pH 7.4), and resuspended in PBS buffer containing 0.5 mM CaCl$_2$, 1 mM MgCl$_2$, and 30 mM glucose (PBS+). Cells were either immediately used for assays or kept on ice for no longer than 3 h. For some experiments (those involving assays for GSH, caspase-3 activity, aminophospholipid externalization, annexin V binding assay, nuclear morphology, and DNA laddering), cells were washed and resuspended in PBS-free RPMI 1640 medium with 10% phenol red. After centrifugation of cells (2 x 10$^6$ cells/ml PMA, an equal volume of phenol red-free RPMI 1640 medium supplemented with 25% FBS (to yield a final concentration of 12.5% FBS) was added to cells, and incubation was extended for another 2 or 4 h. At the end of this period, cells were recovered and washed once with serum-free RPMI 1640 medium. When tested, inhibitors (20 μM DPI, 0.1 μg/ml staurosporine, 50 units/ml SOD plus 50 units/ml catalase (1000 units/ml for neutrophile), and 50 μM z-VAD-fmk) were included in the incubation mixture 5–30 min prior to PMA treatment.

Both Me$_2$SO-differentiated HL-60 cells and neutrophils were stimulated by 50 μg/ml zymosan (opsomized in human or autologous serum, for neutrophil experiments, at 37 °C for 20–30 min) in RPMI medium for 2–6 h. Neutrophils were also stimulated with 0.1 μg/ml PMA. In all experiments, cell viability after treatments was greater than 90%, as assessed by the trypan blue dye exclusion method.

Macrophages (J774A.1; ATCC cell line) were grown in Dulbecco’s modified Eagle’s medium’s supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin sulfate and incubated in a humidified atmosphere (5% CO$_2$ plus 95% air) at 37 °C. Neutrophils were isolated from blood obtained from adult blood donors as described by Faddeel et al. (17). Cells (1.0 x 10$^6$ cells/ml) were maintained in RPMI 1640 medium supplemented with 10% FBS and penicillin plus streptomycin. Following PMA treatment, neutrophils were recovered by trypsinization according to standard procedures; for zymosan-treated cells, no trypsinization was necessary.

**NADPH Oxidase-induced PS Oxidation/Apoptosis**

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Quantification of NADPH Oxidase Activity

After stimulation of cells with PMA, NADPH oxidase activity was determined as the superoxide (O2) production by two different methods: MCLA-enhanced chemiluminescence (to measure instant O2 production at any given moment) and cytochrome c reduction (to measure cumulative amounts of O2 produced over a period of time). MCLA-enhanced Chemiluminescence—MeSO4-differentiated and native HL-60 cells (2 x 10⁶ cells/ml) in prewarmed PBS + were incubated continuously for 1 min in Luminescent Analyzer 633 (Coral Biomedical Inc., San Diego, CA) set at 37 °C and continuous mixing, in the presence of 4 μM MCLA. After 1 min, a stimulant (0.125 μg/ml PMA or 50 μM/ml zymosan) was added by automated injection, and continuous readings were taken for another 10–30 min. Assays were performed in the absence and in the presence of various inhibitors (SO4/catalase, z-VAD-fmk, staurosporine, and z-VAD-fmk). At the end of the incubation period, phospholipid oxidation was determined according to previously described methods (20).

High Performance Thin Layer Chromatography (HPTLC) of Phospholipids

The phospholipid classes in the lipid extract (50 μg of total phospholipids) were separated by two-dimensional HPTLC on silica G plates (5 x 5 cm; Whatman) according to methods previously described (21).

Fluorescamine Labeling of Externalized Aminophospholipids

Labeling of externalized aminophospholipids, PS and phosphatidylethanolamine (PE), with fluorescamine (a nonpermeating probe for visualizing lipids that contain primary amino groups) was carried out by a slight modification of methods previously described by our laboratory (22). Briefly, HL-60 cells (3 x 10⁶) treated with PMA from 0.5 to 2.5 h were suspended in labeling buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM NaHCO3, 5 mM glucose, and 20 mM HEPES; pH 7.4), cells were gently mixed in the presence of fluorescamine (200 μM) for 15 s. The reaction was stopped by the addition of 40 mM Tris-HCl, pH 7.4. Cells were collected by centrifugation, and lipids were extracted by the Folch procedure (23) and analyzed by HPTLC. Fluorescamine-modified PS and PE were localized by exposure of HPTLC plates to UV light by using a Fluor-S™ Multimager (Bio-Rad) imaging system. Ammonium phosphocholine and modified phosphocholine were visualized under visible light in a Fluor-S™ Multimager (Bio-Rad) imaging system after exposure of HPTLC plates to iodine vapor. The phosphorus content of phospholipids was determined according to Bottcher et al. (24) after scraping representative spots from the plate. The amounts of modified PS and PE were expressed as percentages of the total PS and PE (unmodified plus modified) recovered from the plate on the basis of phosphorus content assay.

Flow Cytometric Analysis of PS Externalization

Annexin V binding to cells was determined using a commercially available staining kit (Oncogene Research Products, Boston, MA) and flow cytometry as previously described (22). PMA-stimulated cells were washed once with PBS. Cells were incubated with fluorescein isothiocyanate-conjugated annexin V (0.5 μg/ml) for 15 min and then were collected by centrifugation and washed with binding buffer. Propidium iodide (0.6 μg/ml) was added, and cells were immediately analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) with simultaneous monitoring of green fluorescence (530 nm, 30-nm band pass filter) for annexin V, fluorescein isothiocyanate, and red fluorescence (long pass emission filter that transmits light >650 nm) associated with propidium iodide. A time course for PS externalization was carried out at 0, 0.5, 2.5, and 4.5 h after the addition of PMA or opsonized zymosan to cells. For neutrophils, PS externalization was assessed at 3 and 6 h after the addition of PMA and zymosan, respectively.

Determination of Caspase-3 Activity

The activity of caspase-3 was determined as described previously (25). Briefly, at the indicated times (30 min, 2.5 h, and 4.5 h) after stimulation with PMA or opsonized zymosan, 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, and 5 mM dithiothreitol. The suspensions were centrifuged at 4 °C, and the supernatants were used for analysis. For measurement of caspase activity, 10 μg of lysis diluted to 20 μl with lysis buffer was mixed with 20 μl of 2× ICE buffer (40 mM HEPES-KOH (pH 7.4), 20% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 4 mM dithiothreitol) containing 40 μM acetyl-Asp-Glu-Val-Ala-Asp-7-amino-4-methylcoumarin (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 Corp.) fluorescence microplate reader using excitation at 360 ± 40 nm and emission at 460 ± 40 nm. One unit of caspase activity was defined as the amount of enzyme required to release 1 pmol of 7-aminon-4-methylcoumarin/min. The protein concentration of 10 μg of cell lysates was measured by the method of Bradford (26). For neutrophil experiments, Asp-Glu-Val-Asp-7-amino-4-methylcoumarin data are presented as pmol of 7-amino-4-methylcoumarin released per 10⁶ cells, as previously described (17).
At specified time intervals, commensurate aliquots of PMA-stimulated HL-60 cell suspension were taken, and cells were washed and resuspended in PBS. Hoechst 33342 (5 μg/ml) was added, and cells were examined under fluorescence microscopy. 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 (≥200 cells/time point).

**Determination of Apoptotic Nuclear Morphology**

Macrophage J774A.1 cells were used for phagocytosis assays. Before adding target (naive or Me2SO-differentiated HL-60) cells, macrophages were seeded into an eight-well chamber slide (5 × 10⁴ cells/well) and cultured overnight.

PMA-stimulated cells were washed with serum-free RPMI medium without phenol red and fluorescently labeled with Hoechst 33342 (1 μg/ml). 10 min at 37 °C) and subsequently washed again (three times) with PBS buffer.

Fluorescently labeled cells (5 × 10⁴ cells/well) were added to macrophages, and the mixture was incubated for 1 h at 37 °C. After incubation, unbound target cells were washed three times with RPMI medium and three times with PBS; well contents were fixed with solution of 2% formaldehyde in PBS for 30 min at room temperature. The cells were examined under a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu CCD camera (C4742-95-12NBR) and analyzed using the MetaImaging Series™ software version 4.6 (Universal Imaging Corp., Downingtown, PA). A minimum of 300 macrophages were analyzed per experimental condition.

Results were expressed as the percentage of the phagocytosis-positive macrophages.

For the phagocytosis assay, macrophages that had side-by-side connection with target cells (binding) and/or internalized target cells (engulfment) were considered phagocytosis-positive. To avoid errors due to projections in engulfment assessments, we controlled for counted phagocytosed cells by changing the focusing distance. Use of fluorescent labeling (Hoechst 33342 for target cells) along with bright field analysis of typical macrophage and Me2SO-differentiated HL-60 cell morphologies assured our counting only the macrophages with bound or engulfed HL-60 cells as phagocytosis-positive.

**Statistical Evaluations**

Data are expressed as mean ± S.E. Changes in variables for different assays were analyzed either by Student’s t test (single comparison) or by one-way analysis of variance for multiple comparisons. If any analysis of variance revealed significant changes among samples, multiple unpaired Student’s t tests were performed. Differences were considered to be significant at p < 0.05.

**RESULTS**

**Superoxide and Hydrogen Peroxide Production after Activation of the NADPH Oxidase in Me2SO-differentiated HL 60 Cells**

Results of the online assay of MCLA-enhanced chemiluminescence measuring the rate of O₂⁻ production are shown in Fig. 1A. The rate of O₂⁻ production increased during the first 5–10 min after PMA stimulation of Me2SO-differentiated HL-60 cells and then decayed until it reached base-line levels 30 min after stimulation. The inhibition of chemiluminescence by SOD indicates that the signal was due to O₂⁻ (Fig. 1A, inset). The O₂⁻ production can be attributed to the activation of the NADPH oxidase complex as evidenced by data from three independent experiments: (i) lack of activation in nondifferentiated HL-60 cells, which have no active NADPH oxidase complex (13); (ii) inhibition with staurosporine, a protein kinase C inhibitor that inhibits PMA activation of the NADPH oxidase through a protein kinase C-dependent mechanism; and (iii) inhibition by DPI, a flavoprotein inhibitor that attacks one of the components of the NADPH oxidase complex, flavocytochrome b₅₅₈ (27).

The cytochrome c reduction assay documents the total amount of O₂⁻ produced in the course of incubation. Therefore, the curve of cytochrome c reduction (Fig. 1B) is, in the first approximation, an integral of its first derivative (measure of instant O₂⁻ production at any given moment) shown in Fig. 1A. Our results showed that ~18 nmol O₂⁻/10⁶ cells was generated during the 30-min incubation of Me2SO-differentiated HL-60 cells with PMA (Fig. 1B). A lack of response in nondifferentiated HL-60 cells and inhibition of cytochrome c reduction by either DPI or staurosporine (not shown) further supports the role of the NADPH oxidase.

Amplex Red determination of H₂O₂ production in Me2SO-differentiated HL-60 cells showed its significant stimulation by PMA to yield ~3.6 nmol of H₂O₂/10⁶ cells during 30 min of incubation (after subtraction of the background level) (Fig. 2). Comparison with the data on O₂⁻ production (Fig. 1B) revealed that ~20% of superoxide was detectable as H₂O₂. Formation of H₂O₂ was completely suppressed by DPI, antioxidant enzymes, SOD/catalase, also dramatically decreased PMA-induced levels of H₂O₂. Notably, a pancaspase inhibitor, z-VAD-fmk, signifi-
cantly inhibited PMA-stimulated \( \text{H}_2\text{O}_2 \) production, suggesting that some generated \( \text{H}_2\text{O}_2 \) was associated with the execution of the apoptotic program. This is in line with our data on selective inhibition of PMA-induced PS peroxidation in Me\( \text{2SO} \)-differentiated HL-60 cells (see below).

**NADPH Oxidase-induced GSH Depletion**

Activation of the NADPH oxidase by PMA in Me\( \text{2SO} \)-differentiated HL-60 cells yielded a statistically significant (20\%) decrease in GSH content (Fig. 3A). This depletion was completely reversed when NADPH oxidase activation was inhibited by pretreatment with either DPI or staurosporine. Significant protection was also afforded by SOD/catalase (\( \sim 10\% \) GSH-oxidized), implying that SOD and catalase at these concentrations provide partial protection against oxidative stress induced by PMA (Fig. 3A). Analysis of the sulfhydryl groups associated with proteins revealed no changes under any of the assay conditions tested (Fig. 3B). No changes in GSH content were observed in nondifferentiated HL-60 cells treated with PMA, supporting the link between GSH depletion and NADPH oxidase activation (not shown).

**NADPH Oxidase-induced Phospholipid Peroxidation without Alteration of Phospholipid Composition in Cells**

All three major membrane phospholipids (phosphatidylcholine (PC), PE, and PS) underwent substantial PMA-induced peroxidation in Me\( \text{2SO} \)-differentiated HL-60 cells (Fig. 4). To establish whether oxidation of any particular class of phospholipids was associated with the execution of the apoptotic program, we used a pancaspase inhibitor, z-VAD-fmk. Initially, we determined whether z-VAD-fmk in fact inhibited PMA-induced caspase-3 activation. To this end, we measured the activity of caspase-3 and found that it decreased to background level when Me\( \text{2SO} \)-differentiated HL-60 cells were stimulated by PMA in the presence of 50 \( \mu \)M z-VAD-fmk (Fig. 5). Importantly, only one phospholipid, PS, was protected by z-VAD-fmk against peroxidation (to \( \sim 96\% \) of PnA-PS content in control cells). This indicates that oxidation of PS was most likely specifically associated with PMA-induced apoptosis, whereas oxidation of other phospholipids (PC and PE) was probably due to PMA-dependent activation of the NADPH oxidase and subsequent nonspecific oxidative stress. Phospholipid oxidation was not detected when cells were pretreated either with DPI or with staurosporine at concentrations that inhibited NADPH oxidase activity. Furthermore, when cells were incubated with SOD/catalase, no phospholipid oxidation was observed after PMA stimulation. PMA did not induce any phospholipid oxidation in nondifferentiated HL-60 cells (not shown).

It should be noted that PnA-labeled phospholipids represent a small fraction (1–3\%) of the total amount of phospholipids present in the cell. As a result, massive oxidation of PnA-labeled PS represented a relatively low level of oxidation of...
caspase-3 activity in the absence (control) and in the presence of 0.125 µM PMA was measured 2.5 h after the beginning of treatments. *Inset,* time course of caspase-3 activity in the absence (control) and in the presence of 0.125 µM PMA (T represents the beginning of treatment, and S represents the addition of serum-containing medium). Data are mean ± S.E. (n = 4–8). a, p < 0.005 versus control; b, p < 0.05 versus control (no PMA); c, p < 0.05 versus PMA.

total PS; this was also the case for other phospholipids. Stimulation of Me2SO-differentiated HL-60 cells by PMA did not alter the composition of their phospholipids either relative to control (nonstimulated) cells or relative to time after PMA stimulation (Table I).

Externalization of PS Induced by Activation of the NADPH Oxidase

A selective redistribution of PS in plasma membrane from cells treated with PMA was observed as an increased proportion of fluorescamine-reactive PS on the cell surface, which escalated over time (Fig. 6A). The concurrent (nonstimulated) control group did not show any change from the background level of externalized PS; after 30 min of PMA treatment, cells exhibited an increase from 2.6 to 11.5% of externalized PS (Fig. 6A). Whereas the level of PS exposed on the cell surface of PMA-treated cells reached ~16.6% 2 h later, the control group showed only a slight increase to 6.1% (Fig. 6A). This demonstrates that PS was externalized and became accessible to fluorescein in the extracellular leaflet of plasma membrane after exposure to PMA. Some amount of PE is exposed on the surface of normal cells and hence should be available for fluorescein. Indeed, we found that ~6.3% of total PE was reactive toward fluorescein in normal Me2SO-differentiated HL-60 cells (Fig. 6A, inset). The amount of fluorescein-reactive PE was increased almost 3-fold after 0.5 h of incubation in serum-free RPMI medium as well as 2.5 h of incubation. Most importantly, PMA stimulation did not significantly affect PE externalization, as observed by this assay as compared with its amounts available for fluorescein in nonstimulated cells. Thus, no NADPH oxidase-dependent PE externalization was detectable in Me2SO-differentiated HL-60 cells.

Although the fluorescein-based assay allows quantification of the total amount of PS externalized in the cell suspensions, it does not provide any information about how these PS molecules are distributed in the cell population. The use of annexin V, a molecule that specifically binds to PS, together with flow cytometry permitted us to address this question. We observed a time-dependent increase in the proportion of cells with externalized PS (Fig. 6B). At 4 h after PMA stimulation, 70% of cells externalized PS on the outer leaflet of plasma membrane. Approximately 49% of these PS-externalizing cells were annexin V/PI−; 51% of the cells were annexin V/PI+. At an earlier time point (30 min of PMA stimulation), 40% of cells were either annexin V/PI− or annexin V/PI+.

Most of the cells (~62%) with externalized PS retained an intact membrane (annexin V/PI−). Pretreatment of cells with DPI or staurosporine almost completely abrogated PS externalization after activation of the NADPH oxidase by PMA (Fig. 5C). The combination of SOD/catalase partially inhibited such externalization of PS (Fig. 5C). These results clearly support an association between activation of the NADPH oxidase and PS externalization on the surface of Me2SO-differentiated HL-60 cells as well as the involvement of ROS in this process.

**NADPH Oxidase-induced Expression of Biomarkers of Apoptosis**

To provide evidence for NADPH oxidase-induced apoptosis upon PMA stimulation of Me2SO-differentiated HL-60 cells, several biomarkers of apoptosis were assayed.

**Activation of Caspase-3**—After stimulation of the cells with PMA, activation of caspase-3 was observed for as long as 4.5 h (Fig. 5), showing significant activation 2.5 h after PMA exposure (Fig. 5, inset). Furthermore, treatment of cells with SOD/catalase significantly inhibited the PMA-induced activation of caspase-3. Expectedly, caspase-3 activation was sensitive to z-VAD-fmk (50 µM). Inhibition of the NADPH oxidase by either DPI or staurosporine almost completely blocked caspase-3 activity (Fig. 5). Nondifferentiated HL-60 cells showed no activation of caspase-3 upon stimulation by PMA (not shown).

**Chromatin Condensation and Nuclear Fragmentation**—Microscopic examination of nuclear morphology showed that, after exposure to PMA, an increasing percentage of Me2SO-differentiated HL-60 cells exhibited nuclear condensation and fragmentation, typical characteristics of apoptosis (Fig. 7). Inhibition of the NADPH oxidase by either staurosporine or DPI and scavenging of O2−/H2O2 by the SOD/catalase system abrogated the nuclear changes associated with NADPH oxidase activation. Nondifferentiated HL-60 cells did not show any change in nuclear morphology after stimulation by PMA (not shown). DNA from Me2SO-differentiated HL-60 cells treated with PMA also displayed “laddering,” a typical hallmark of apoptosis (data not shown).

**Activation of the NADPH Oxidase Stimulates Phagocytosis of Me2SO-differentiated HL-60 Cells by J774A.1 Macrophages**—Exposure of PS on the surface of the plasma membrane acts as a distinctive signal that allows macrophages to recognize PS-externalized cells and remove them from surrounding tissues (1–6). The percentage of macrophages recognizing and/or phagocytizing PMA-stimulated Me2SO-differentiated HL-60 cells was ~23%, a significant increase when compared with the 10% of phagocytosis-positive macrophages observed after coculture with nonstimulated Me2SO-differentiated HL-60 cells (Fig. 8). When the NADPH oxidase was inhibited by either DPI or staurosporine, the percentage of recognizing and/or phagocytizing macrophages decreased to values observed when non-PMA-stimulated Me2SO-differentiated HL-60 cells were added to macrophage cultures (Fig. 8). Treatment of Me2SO-differentiated HL-60 cells with SOD/catalase showed a significant inhibitory effect on phagocytosis (Fig. 8). After exposure to PMA, nondifferentiated HL-60 cells were not phagocytized by J774A.1 macrophages (not shown).

**Zymosan Stimulation of the NADPH Oxidase and Oxidative Stress in Me2SO-differentiated HL-60 Cells**

We further used opsonized zymosan to determine whether similar PS-dependent responses could be observed in Me2SO-
NADPH Oxidase-induced PS Oxidation/Apoptosis

Table I

Phospholipid composition of MeSO-differentiated HL-60 cells (percentage of total) by HPTLC

MeSO-differentiated HL-60 cells (2 × 10^6 cells/ml) were incubated in the absence (control) or in the presence of 0.125 μg/ml PMA in FBS-free RPMI 1640 medium without phenol red for 0.5 h at 37 °C. After this incubation period, an equal volume of RPMI 1640 medium without phenol red supplemented with 25% FBS (to yield a 12.5% final concentration) was added to cells, and incubation was extended for another 2 h. At the indicated times, aliquots were taken, and phospholipid composition was analyzed by HPTLC. Data are means ± S.E. (n = 4).

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control 0 h</th>
<th>Control 0.5 h</th>
<th>Control 2.5 h</th>
<th>PMA treatment 0.5 h</th>
<th>PMA treatment 2.5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylglycerol</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>27.7 ± 1.2</td>
<td>27.1 ± 0.6</td>
<td>26.8 ± 0.8</td>
<td>28.2 ± 1.0</td>
<td>28.2 ± 1.0</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>45.8 ± 1.5</td>
<td>47.9 ± 1.0</td>
<td>46.5 ± 0.8</td>
<td>46.0 ± 0.7</td>
<td>43.9 ± 1.7</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>10.9 ± 0.9</td>
<td>9.9 ± 0.2</td>
<td>10.2 ± 0.6</td>
<td>11.0 ± 0.2</td>
<td>11.1 ± 1.5</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>6.8 ± 0.5</td>
<td>8.4 ± 0.5</td>
<td>6.7 ± 0.5</td>
<td>6.3 ± 0.7</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>6.7 ± 0.5</td>
<td>6.2 ± 0.4</td>
<td>7.4 ± 0.3</td>
<td>7.5 ± 0.3</td>
<td>8.5 ± 0.6</td>
</tr>
</tbody>
</table>

Fig. 6. Externalization of PS induced by PMA in MeSO-differentiated HL-60. MeSO-differentiated HL-60 cells (2 × 10^6 cells/ml) were incubated in the absence (control) or in the presence of 0.125 μg/ml PMA in FBS-free RPMI 1640 medium without phenol red for 0.5 h at 37 °C. After this incubation period, an equal volume of phenol red-free RPMI 1640 medium supplemented with 25% FBS (to yield a 12.5% final concentration) was added to cells, and incubation was extended for another 2 or 4 h. At the indicated times, an aliquot was taken, and phospholipid externalization was analyzed by the following methods. A, fluororescamine-associated fluorescence and HPTLC of externalized PS. The same procedure was carried out for monitoring externalization of PE, shown as the inset. Data are mean ± S.E. (n = 4). a, p < 0.02 versus control, 0.5 h; b, p < 0.02 versus control, 2.5 h. B and C, annexin V binding assay and flow cytometry. B, time course for PS externalization. Data represent values from PMA-treated minus control cells, expressed as mean ± S.E. (n = 4–8); c, p < 0.001 versus the previous time point; d, p < 0.01 versus the previous time point. C, effect of SOD/catalase, DPI, and staurosporine on PS externalization. Data are expressed as mean ± S.E., n = 3. e, p < 0.001 versus the rest of treatments; f, p < 0.001 versus control.

differentiated HL-60 cells with physiologically more relevant phagocytic stimuli. We found that serum-oposinized zymosan caused activation of caspase-3 in MeSO-differentiated HL-60 cells that was completely blocked by a pancaspase inhibitor, z-VAD-fmk (Fig. 9A). Zymosan induced a pronounced production of superoxide in HL-60 cells (Fig. 9B). Whereas zymosan-induced oxidative burst was ~3-fold less than that induced by PMA, it was also partially inhabitable by z-VAD-fmk and completely blocked by SOD/catalase as well as by DPI (data not shown). Again, similar to PMA, opsonized zymosan caused a significant oxidation of different classes of phospholipids (Fig. 9C). Remarkably, only PS oxidation was significantly inhibited by z-VAD-fmk (from 31.0 ± 3.9% of PS oxidized in the absence of z-VAD-fmk to 16.0 ± 3.7% of PS in the presence of z-VAD-fmk), whereas oxidation of other major phospholipids such as PC and PE was not significantly changed by the pancaspase inhibitor. We also observed that zymosan caused PS externalization in MeSO-differentiated HL-60 cells (~44% of cells were annexin V+/PI−). PS externalization was partially suppressed by SOD/catalase and to a lesser extent by z-VAD-fmk (data not shown). Thus, both PMA and opsonized zymosan caused oxidative stress and PS oxidation in MeSO-differentiated HL-60 cells associated with the execution of the apoptotic program as revealed by the sensitivity toward z-VAD-fmk.

PMA- and Zymosan-induced NADPH Oxidase Activation and PS Externalization in Neutrophils

Finally, we performed an additional series of experiments with human neutrophils stimulated with PMA or serum-oposinized zymosan. We found that no caspase-3 activation was induced in PMA-treated cells in line with previous observations (17). Zymosan treatment, on the other hand, markedly enhanced caspase-3 activation when compared with constitutive, background levels (Fig. 10A). This effect was not pronounced at 3 h (data not shown), but was seen clearly at later time points.
**DISCUSSION**

Neutrophils possess powerful ROS-generating machinery, the NADPH oxidase system, to kill invading microorganisms. Uncontrolled activation of neutrophils in anomalous inflammatory processes promotes different oxidation-associated injuries in several acute conditions, including acute respiratory distress syndrome and reperfusion injury, and in chronic diseases such as emphysema, atherosclerosis, and rheumatoid arthritis (for a review, see Ref. 28). Preeclampsia, a human pregnancy-related disorder, has been also associated with neutrophil activation (29, 30) and with delay of neutrophil apoptosis (31) as sources of the high level of oxidative stress.

Activated neutrophils should be removed from inflamed areas once invading microorganisms have been eliminated and the infection has been resolved. This removal takes place through recognition of apoptotic neutrophils by phagocytes via a pathway that involves exposure of PS on neutrophil cell surfaces (1–6). Yet the mechanisms underlying apoptosis in activated neutrophils, particularly those involving PS externalization, are not fully characterized.

**NADPH Oxidase-induced Oxidative Stress and Apoptosis in Me2SO-differentiated HL-60 Cells**—In the present work, we have demonstrated that when the NADPH oxidase was activated by PMA or opsonized zymosan in neutrophil-like Me2SO-differentiated HL-60 cells, apoptosis was increased. Furthermore, the increase was correlated with enhanced phagocytosis by macrophages. Our data further show that activation of the NADPH oxidase complex in neutrophil-like cells triggers the mechanisms necessary for phagocytic clearance of neutrophils from inflamed areas by mechanisms involving oxidative stress.

Several authors have observed no induction of apoptosis in Me2SO-differentiated HL-60 cells after stimulation with PMA and have attributed this observation to the lack of specific granules and, therefore, of intracellular production of H2O2 in the cells (9). Neutrophils, however, release oxidants extracellularly.
NADPH Oxidase-induced PS Oxidation/Apoptosis

A fraction of O$_2^-$ is produced during NADPH oxidase activity and can react with NO to form peroxynitrite (ONOO$^-$). This reactive species is capable of inducing oxidative stress and apoptosis. To facilitate removal of ROS during PMA-induced oxidative stress and apoptosis, we used a combination of SOD and catalase. Since both enzymes remain in the extracellular compartment, they can only eliminate extracellular O$_2^-$ and H$_2$O$_2$. A fraction of O$_2^-$ and H$_2$O$_2$ generated intracellularly (e.g., during execution of the apoptotic program as a result of electron transport disruption after departure of cytochrome c from mitochondria) will probably be unavailable for exogenously added SOD/catalase. H$_2$O$_2$, however, can diffuse from cells and hence become available for extracellular catalase. Therefore, we utilized a combination of SOD (to scavenge any excreted superoxide anions and convert them into H$_2$O$_2$) and catalase (to decompose extracellular H$_2$O$_2$ produced either extracellularly or intracellularly). SOD/catalase at the concentrations used showed only partial protection against oxidative stress and apoptosis as evidenced by partial inhibition of H$_2$O$_2$ production and GSH oxidation as well as caspase-3 activation and PS externalization.

Some intracellular H$_2$O$_2$ could have been available to produce oxidative stress (e.g., oxidize GSH through an enzymatic mechanism involving glutathione peroxidase). Depletion of GSH induces neutrophil apoptosis through a mechanism dependent on caspase-3 activation (36). In our experiments, the partial protection of SOD/catalase against GSH oxidation is in agreement with concomitant partial activation of caspase-3 activity; one explanation for this partial effect of the antioxidant enzymes is that their concentrations might be insufficient.

**Oxidation of PS and Phospholipid Signaling in Me$_2$SO-differentiated HL-60 Cells**—Here we propose a new role for oxidative stress in phospholipid signaling through oxidation of PS and its further externalization. We observed that all three major membrane phospholipids, PC, PE, and PS, underwent significant PMA-induced peroxidation in Me$_2$SO-differentiated HL-60 cells. In these cells, PMA induces oxidation through two different mechanisms: (i) via stimulation of the NADPH oxidase that generates ROS and, hence, catalyzes oxidation of different cellular constituents, including phospholipids, and (ii) due to triggering and execution of the apoptotic program. One may assume that the former pathway will be associated with nonspecific oxidation of all major classes of phospholipids,
whereas the latter may be specific to those involved in oxidative signaling pathways of apoptosis. To differentiate between these two mechanisms, we used a pancaspase inhibitor, z-VAD-fmk, that blocked PMA-induced caspase-3 activity in MeSO-differentiated HL-60 cells. We found that, indeed, only PS was protected by z-VAD-fmk against PMA-induced oxidation, whereas two more abundant phospholipids, PC and PE, were oxidized to the same extent in the absence or presence of z-VAD-fmk. In contrast, inhibitors of NADPH oxidase activity, DPI and staurosporine, blocked oxidation of all phospholipids to the same degree. Similarly, antioxidant enzymes, SOD/catalase, protected all three phospholipids against peroxidation. Taken together, these results strongly suggest that PS oxidation is likely to represent an apoptosis-specific event. This is further supported by partial inhibition of PMA-induced H$_2$O$_2$ production by z-VAD-fmk in MeSO-differentiated HL-60 cells.

We further used opsonized zymosan to determine whether similar PS-dependent responses could be observed in MeSO-differentiated HL-60 cells with physiologically more relevant phagocytosable stimuli. Opsonized zymosan activated the NADPH oxidase and induced apoptosis, as evidenced by superoxide anion production, PS externalization, and caspase-3 activation. Furthermore, we observed the same phospholipid oxidation pattern with zymosan as with PMA. Thus, both PMA and opsonized zymosan caused oxidative stress in MeSO-differentiated HL-60 cells, part of which was associated with the execution of apoptotic program, as revealed by the sensitivity toward z-VAD-fmk.

Non-oxidant-induced apoptosis may be a preferable model for the elucidation of phospholipid oxidative signaling mechanisms. Therefore, in a separate study, we utilized a model of staurosporine-induced apoptosis in HL-60 cells in which treatment with staurosporine caused marked oxidation of PS while PC remained unoxidized (37). In addition, employing another model of non-oxidant-induced apoptosis (death receptor triggering using agonistic anti-Fas antibodies), we have recently reported production of both superoxide (detected by MCLA chemiluminescence response) as well as H$_2$O$_2$ (detected by Amplex Red assay) in Jurkat T cells (38). Both superoxide generation and accumulation of H$_2$O$_2$ were substantially attenuated by z-VAD-fmk. Most notably, PS was preferentially oxidized upon Fas ligation (38).

Oxidized epitopes on the surface of apoptotic cells are known to be important signals for the recognition of target cells by macrophages (39–40). To verify the importance of oxidized PS in phagocytosis of apoptotic cells, we used nonapoptotic HL-60 cells with PS or with PS plus oxidized PS integrated in the outer surface of their plasma membrane (38). We found that a mixture of PS plus oxidized PS yielded an almost 2-fold enhancement of PS-dependent phagocytosis of HL-60 cells by J774A.1 macrophages. Conversely, pretreatment of macrophages with liposomes containing a mixture of PS plus oxidized PS inhibited phagocytosis of apoptotic HL-60 cells more effectively than pretreatment with liposomes loaded with nonoxidized PS alone.

In the current study, phospholipid peroxidation did not affect overall phospholipid composition in MeSO-differentiated HL-60 cell membranes. Rather, NADPH oxidase-dependent oxidation of PS altered the usual distribution of PS in resting, nonstimulated cells and resulted in externalization of PS on the cell surface (9–10% more externalized PS than in the control group). The most commonly used assay for assessment of PS externalization, based on the interactions of PS with annexin V on the cell surface, is not absolutely specific. Other negatively charged moieties including peroxidation products of aminophospholipids can bind annexin V as has been recently reported by Balasubramanian et al. (41). It should be kept in mind, however, that these interactions occur at relatively high concentrations of aminophospholipid peroxidation products, hardly achievable in living cells (after exposure of cells to ~5 mM malonyl dialdehyde), including cells executing the apoptotic program. Our PNA-based assay of phospholipid peroxidation indicates that the total concentration of oxidatively modified phospholipids did not exceed 3–5 µM (i.e., was about 3 orders of magnitude lower than that used by Balasubramanian et al. (41) for cell treatments). The amount of oxidatively modified aminophospholipids on the cell surface is likely to be even smaller. Therefore, binding of annexin V to aminophospholipid peroxidation products on the surface of apoptotic cells does not seem to substantially affect measurements of PS externalization.

Oxidation of PS may stimulate externalization of both PS and oxidized PS. There are different models explaining specific mechanisms through which oxidation of PS may facilitate externalization of PS or oxidized PS. According to one such model, oxidized PS poisons the aminophospholipid translocase, the enzymatic activity that is responsible for the maintenance of membrane plasma phospholipid asymmetry (42), in which case both PS and oxidized PS would be expected to appear on the cell surface. Another scenario is that the aminophospholipid translocase fails to recognize oxidized PS, resulting in selective externalization of oxidized PS. In the latter case, inhibition of PS oxidation should result in complete inhibition of PS externalization. In the former case, inhibition of PS oxidation may be consistent with the presence of PS (but not oxidized PS) on the cell surface. However, we do not have any information on the amounts of oxidized PS on the surface of apoptotic cells. Oxidation of phospholipids may also occur in the outer leaflet of the membrane by extracellular oxidants such as H$_2$O$_2$. In this case, prevention of externalization by caspase inhibition (z-VAD-fmk) would therefore prevent oxidation of PS but not of PE or PC. Further studies are necessary to establish the link between PS oxidation and externalization of oxidized or nonoxidized PS molecular species.

**NADPH Oxidase-dependent Externalization of PS in Human Neutrophils**—In the current study, we chose to use MeSO-differentiated HL-60 cells as a well defined neutrophil-like model cell line that can be readily maintained in standard conditions and to use a well known stimulus (PMA) to activate the NADPH oxidase for gaining insight into possible involvement of phospholipid oxidation in signaling pathways. However, our observations in MeSO-differentiated HL-60 cells may not be directly applicable to the in vivo situation. Therefore, we also performed experiments using freshly isolated human neutrophils. Our data demonstrate that NADPH oxidase-induced oxidative stress is involved in PS externalization in PMA- or zymosan-stimulated neutrophils. In a manner consistent with our observations, PMA treatment of neutrophils obtained from patients with chronic granulomatous disease that lack a functional NADPH oxidase does not result in PS externalization (17).

We have previously reported that PMA was able to activate caspase-3 in the presence (but not in the absence) of the NADPH oxidase inhibitor, DPI (17). This is most likely due to oxidative inactivation of the cysteine-dependent caspases upon PMA treatment of these cells (43). Thus, two processes (PMA-induced activation of caspase-3 and inactivation of caspase-3 by NADPH oxidase-dependent ROS) may occur simultaneously in activated neutrophils. This suggests that suppression of NADPH oxidase ROS production prevents oxidative inactivation of caspase-3 and is compatible with detectably enhanced levels of caspase-3 in neutrophils. In our current experiments,
PMA caused a time-dependent increase of caspase-3 activity in Me₃SO-differentiated HL-60 cells. This activity was completely inhibitable by a pancaspase inhibitor, z-VAD-fmk, as well as by inhibitors of NADPH oxidase activity, DPI and staurosporine, and antioxidant enzymes, SOD/catalase. These results indicate that PMA-induced activation of caspase-3 in Me₃SO-differentiated HL-60 cells is not as redox-sensitive as in activated neutrophils. This may be due, at least in part, to a lower level of ROS production by PMA-stimulated Me₃SO-differentiated HL-60 cells as compared with neutrophils. Indeed, it has been demonstrated that Me₃SO-differentiated HL-60 cells was 7–8-fold lower than by activated neutrophils (18).

Since PMA does not trigger a typical caspase-dependent program of cell death in neutrophils (17) (the present study), we chose to use oposized zymosan as a more physiologically relevant stimulus. Zymosan triggered superoxide production in human neutrophils that was ~2.5-fold less pronounced than that induced by PMA. In this case, a significant degree of caspase-3 activation was indeed, observed. Moreover, zymosan-induced caspase activation was suppressed by DPI, thus demonstrating that this event is NADPH oxidase-dependent. Of note, zymosan-induced SOD externalization in neutrophils was also blocked by DPI, SOD/catalase, and to a lesser degree by z-VAD-fmk. These results are in agreement with previously reported data by Coxon et al. (45). That opsonized particles can induce oxidant-dependent and DPI-inhibitable apoptosis in neutrophils.

In conclusion, HL-60 cells differentiated into the neutrophil lineage undergo apoptosis and phagocytosis upon stimulation of the NADPH oxidase by PMA or opsonized zymosan. Furthermore, PS oxidation is likely to represent an apoptosis-specific event, as z-VAD-fmk and SOD/catalase block both PS oxidation and the execution of apoptosis in these cells. Finally, SOD/catalase-inhibitable externalization of PS is important for apoptotic signaling in neutrophils. Moreover, we have shown that NADPH oxidase-dependent, SOD/catalase-inhibitable externalization of PS is important for macrophage clearance of apoptotic neutrophil-like cells. Based on these findings, we thus propose a new role for oxidative stress in phospholipid signaling through oxidation of PS and its further externalization, and we suggest that similar mechanisms may be operating at sites of inflammation to ensure that effete cells are effectively disposed of.

Acknowledgment—We thank Dr. Teresa L. Bradley for valuable help in preparing the manuscript.

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NADPH Oxidase-dependent Oxidation and Externalization of Phosphatidylinerine during Apoptosis in Me$_2$SO-differentiated HL-60 Cells: ROLE IN PHAGOCYTIC CLEARANCE

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J. Biol. Chem. 2002, 277:49965-49975, doi: 10.1074/jbc.M204513200 originally published online October 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204513200

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