Nitrosative Stress Inhibits the Aminophospholipid Translocase Resulting in Phosphatidylserine Externalization and Macrophage Engulfment

IMPLICATIONS FOR THE RESOLUTION OF INFLAMMATION*

Received for publication, July 21, 2006, and in revised form, January 16, 2007 Published, JBC Papers in Press, January 17, 2007 DOI 10.1074/jbc.M606950200

Yulia Y. Tyurina‡, Liana V. Basova, Nagarjun V. Konduru, Vladimir A. Tyurin§, Ala I. Potapovich, Peter Cai‡, Hülya Bayir††, Detcho Stoyanovsky, Bruce R. Pitt†, Anna A. Shvedova, Bengt Fadeel**, and Valerian E. Kagan††††§§§

From the ‡Center for Free Radical and Antioxidant Health, Departments of Environmental and Occupational Health, Pharmacology, Critical Care Medicine, Surgery, and §§§Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania 15219, †National Institute of Occupational Safety and Health, Morgantown, West Virginia 26505, and **Division of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institutet, 17177 Stockholm, Sweden

Macrophage recognition of apoptotic cells depends on externalization of phosphatidylserine (PS), which is normally maintained within the cytosolic leaflet of the plasma membrane by aminophospholipid translocase (APLT). APLT is sensitive to redox modifications of its –SH groups. Because activated macrophages produce reactive oxygen and nitrogen species, we hypothesized that macrophages can directly participate in apoptotic cell clearance by S-nitrosylation/oxidation and inhibition of APLT causing PS externalization. Here we report that exposure of target HL-60 cells to nitrosative stress inhibited APLT, induced PS externalization, and enhanced recognition and elimination of “nitrosatively” modified cells by RAW 264.7 macrophages. Using S-nitroso-1-cysteine-ethyl ester (SNCEE) and S-nitrosoglutathione (GSNO) that cause intracellular and extracellular trans-nitrosylation of proteins, respectively, we found that SNCEE (but not GSNO) caused significant S-nitrosylation/oxidation of thiols in HL-60 cells. SNCEE also strongly inhibited APLT, activated scramblase, and caused PS externalization. However, SNCEE did not induce caspase activation or nuclear condensation/fragmentation suggesting that PS externalization was dissociated from the common apoptotic pathway. Dithiothreitol reversed SNCEE-induced S-nitrosylation, APLT inhibition, and PS externalization. SNCEE but not GSNO stimulated phagocytosis of HL-60 cells. Moreover, phagocytosis of target cells by lipopolysaccharide-stimulated macrophages was significantly suppressed by an NO’ scavenger, DAF-2. Thus, macrophage-induced nitrosylation/oxidation plays an important role in cell clearance, and hence in the resolution of inflammation.

Programmed cell death (apoptosis), aimed at harmless elimination of irreparably damaged or unwanted cells, and subsequent removal of apoptotic cells are strongly coupled with the regulation of normal tissue function and structure, both in the developing and adult organism (1–4). Moreover, recognition of apoptotic cells and their clearance by macrophages play an active role in the resolution of inflammation, through production of anti-inflammatory cytokines, down-regulation of pro-inflammatory mediators, and macrophage generation and release of reactive oxygen and nitrogen species (5). Dysregulation and ineffective clearance of apoptotic cells result in post-apoptotic cytolysis (6) and cause pro-inflammatory conditions that are associated with a number of autoimmune and chronic inflammatory diseases such as systemic lupus erythematosus (7), chronic obstructive pulmonary disease (8), chronic granulomatous disease (9), and asthma (3).

Phagocytic recognition of apoptotic cells requires a remarkable rearrangement of their membrane surface. Most notably, collapse of plasma membrane phospholipid asymmetry, appearance of phosphatidylserine (PS),2 an essential “eat-me” signal, on the cell surface (10), and its interactions with specialized binding proteins (11, 12) are involved in recognition, tethering, and engulfment of apoptotic cells by phagocytes (13). Moreover, externalized PS is essential for triggering of anti-inflammatory responses in macrophages (2, 4, 7). Therefore, failure to externalize PS may disrupt the clearance of apoptotic cells in vivo and contribute to perpetuating a calamitous pro-inflammatory environment (14).

Normally PS is confined exclusively to the cytoplasmic surface of the plasma membrane (15); maintenance of this asymmetric PS distribution is because of energy (ATP)-dependent aminophospholipid translocase (APLT) responsible for the inward translocation of amino phospholipids (15, 16). During

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2 The abbreviations used are: PS, phosphatidylserine; NBD-PS, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoazidazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phospho-L-serine; NBD-PC, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoazidazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine; PC, phosphatidylcholine; DTT, dithiothreitol; GSNO, S-nitrosoglutathione; SNCEE, S-nitroso-L-cysteine ethyl ester; LPS, lipopolysaccharide; DAF-2, 4,5-diaminofluorescein; DAF-2DA, 4,5-diaminofluorescein diacetate; DHE, dihydroethidium; O2., superoxide; NO’, nitric oxide; ONOO-, peroxynitrite; SWCNT, single walled carbon nanotubes; APLT, aminophospholipid translocase; ANOVA, analysis of variance; HPTLC, high performance thin layer chromatography; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; Z, benzoyloxy carbonyl; fmk, fluoromethyl ketone.
apoptosis, APLT is inactivated causing egress of PS from the inner to the outer leaflet of the plasma membrane (17–19). The mechanisms of apoptotic APLT inactivation remain to be elucidated. Reportedly, APLT is not a substrate for apoptosis-activated caspases suggesting that alternative mechanisms may be involved (20). In line with this, PS externalization can be divorced from the common caspase-initiated pathway of apoptosis (21, 22). Connor and Schroit (23) demonstrated that reversible PS externalization can be induced by −SH reagents in non-apoptotic cells. Conversely, certain cell types can undergo apoptosis without PS externalization (24, 25), but the resistance to PS externalization in these cells can be completely reversed by treatment with thiol reagents (23, 25). Apparently, the sensitivity of catalytically competent cysteines of APLT to oxidation/alkylation may be important in the regulation of the enzyme activity during apoptosis (5, 16–18, 26).

S-Nitrosylation is another redox-related mechanism participating in the modification of protein cysteines (27). A variety of enzymes contain essential cysteines whose S-nitrosylation results in changed catalytic activity (28). The two major pathways for protein S-nitrosylation are as follows: 1) trans-nitrosylation resulting from the transfer of the nitrosyl functionality from a donor S-nitrosothiol (commonly S-nitrosoglutathione or other low molecular weight thiol) to a recipient cysteine, and 2) direct nitrosylation by peroxynitrite (ONOO⁻) (formed as a product of the reaction between radical species, nitric oxide (NO) and superoxide (O₂⁻)) (29). Importantly, during inflammation, NADPH oxidase and inducible nitric-oxide synthase in activated macrophages massively produce O₂⁻ and NO⁻, respectively, to yield ONOO⁻ (30, 31). The latter can readily S-nitrosylate low molecular thiols (32) and hence generate pools of trans-nitrosylating species ready to attack protein cysteines (27).

We hypothesized that activated macrophages can directly participate in the clearance of target (apoptotic) cells and bystander cells by causing S-nitrosylation/oxidation and inhibition of APLT and inducing PS externalization independently of the execution of the common caspase-dependent pathway of apoptosis. In this study, we demonstrate that exposure of target HL-60 cells to nitrosative stress indeed inhibits APLT, induces PS externalization, and enhances recognition and elimination of “nitosatively” modified cells by RAW 264.7 macrophages. We suggest that this novel mechanism of macrophage-induced nitrosative stress contributes to effective clearance of apoptotic cells and regulates switching of the acute inflammatory reaction to an anti-inflammatory phase of the response.

**EXPERIMENTAL PROCEDURES**

**Reagents**—160–120 NBD-PS, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phospho-l-serine (ammonium salt), and 160–120 NBD-PC, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-Phospho-choline (ammonium salt), were from Avanti Polar Lipids Inc. (Alabaster, AL). Dithiothreitol (DTT), S-nitrosoglutathione (GSNO), dithionite, HEPES, glucose, MgCl₂, CaCl₂, KCl, NaCl, acetonitrile, phenylmethylsulfonyl fluoride, lipopolysaccharide (LPS), and Hoechst 33342 were from Sigma. S-Nitroso-1-cysteine-ethyl ester (SNCEE) was synthesized as described previously (33). DAF-2, DAF-2DA, and ThioGlo™-1 were purchased from Calbiochem. DHE-DA and Cell Tracker Orange were from Molecular Probes (Eugene, OR).

**Culture**—HL-60 human promyelocytic leukemia cells (American Type Culture Collection) were grown in RPMI 1640 with phenol red supplemented with 12.5% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere (5% CO₂ plus 95% air). Cells from passages 45–50 were used for the experiments. The density of the cells at collection time was 0.5 × 10⁶ cell/ml. RAW 264.7 macrophages (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere (5% CO₂ plus 95% air) at 37 °C.

**Measurement of Thiol Contents**—Low molecular weight thiols and protein thiol contents in the cells were determined fluorometrically using ThioGlo™-1 as described previously (18). Briefly, cells treated with either SNCEE (50–300 µM) or GSNO (50–300 µM) for 30 min at 37 °C were collected by centrifugation, washed, and resuspended in PBS. GSH was measured in cell lysates prepared by freezing and thawing cells. Immediately after addition of ThioGlo™-1 to the cell lysates, fluorescence was measured in a Packard Fusion™ Multifunctional Plate Reader (PerkinElmer Life Sciences) using excitation 390 ± 15 nm and emission 515 ± 30 nm. Protein sulfhydryls were determined as an additional increase in fluorescence response after addition of SDS (4 mM) to cell lysates.

**Measurement of S-Nitrosothiols**—The content of S-nitrosothiols was determined by fluorescence HPLC using DAF-2 that specifically reacts with NO⁻ (but not with other NOₓ, ONOO⁻, NO₃⁻, or NO₂⁻), yielding fluorescent DAF-2 triazole (34). Briefly, samples (50 µl) were mixed with DAF-2 (5 µM) in 2.5 ml of PBS and exposed to UV irradiation (15 min at room temperature) using an Oriel UV light source (model 66002) (Oriel Instruments, Stratford, CT) and cut-off filter (Balzers, >330 nm) (35). After UV irradiation, chloroform (200 µl) was added to precipitate proteins, and samples were centrifuged at 14,000 × g for 5 min. Supernatant was used for the measurements. Fluorescence HPLC (Eclipse XDB-C18 column, 5 µm, 150 × 4.6 mm; mobile phase was composed of 25 mM disodium phosphate buffer, pH 7.0, acetonitrile (94.6 v/v), Ex = 495 nm, Em = 515 nm) was performed on a Shimadzu LC-100AT HPLC system equipped with fluorescence detector (RF-10AXl) and autosampler (SIL-10AD). The data obtained were exported and processed using Shimadzu RF-5301 PC personal software. A standard curve was established using GSNO as the standard.

**APLT and Scramblase Activity Assays**—APLT and scramblase activity were measured using NBD-PS and NBD-PC, respectively, as described previously (36, 37). HL-60 cells (5 × 10⁶) were exposed to SNCEE (50–300 µM) or GSNO (50–300 µM) for 30 min at 37 °C in serum-free RPMI 1640 medium without phenol red. At the end of incubation, the cells were washed twice and suspended in ice-cold incubation buffer (136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 5 mM glucose, 500 µM phenylmethylsulfonyl fluoride, 10 mM HEPES, pH 7.5) and incubated with either NBD-PS or NBD-PC (10 µM) for 10 min.
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at 4 °C. Then the cells were pelleted by centrifugation, resuspended in the same buffer (5 × 10^6 cells/ml), and placed in a water bath (at 28 °C) to initiate internalization of NBD-PS. For measurements of scramblase activity, 2 mM CaCl_2 was added to the incubation medium. A Shimadzu RF-5301PC spectrophotometer was employed for these measurements using the following instrumental conditions: Ex = 470 nm, Em = 540 nm; slits 5 and 10 nm, respectively. We normalized the differences in fluorescence intensities recorded immediately after removal of unbound of NBD-PS by centrifugation and the responses obtained from residual noninternalized NBD-PS (after allowing its internalization for given periods of time) in the presence and absence of a reducing agent, dithionite, to the fluorescence intensity of bound NBD-PS measured in the absence of dithionite; FL<sub>d</sub> is the fluorescence intensity from the cells with NBD-lipid before the initiation of internalization (at 28 °C). Specifically, the percent of internalized NBD-PS was calculated using the following equation:

\[ \text{% internalized NBD-PS} = \left( \frac{\text{FL}_t - \text{FL}_d}{\text{FL}_{\text{total}} - \text{FL}_d} \right) \times 100 \]

where FL<sub>t</sub> is the fluorescence intensity measured in the presence of sodium dithionite (10 mM) at a given time point; FL<sub>total</sub> is the fluorescence response from bound plus internalized NBD-PS measured in the absence of dithionite; FL<sub>d</sub> is the fluorescence intensity from the cells with NBD-lipid before the initiation of internalization but measured in the presence of dithionite.

To estimate possible effects of endocytosis on the uptake of NBD-PS, HL-60 cells were incubated (30 min at 37 °C) in the presence of a mixture containing several commonly used inhibitors of endocytosis (38) as follows: nystatin (25 μg/ml), genistein (200 μM), chlorpromazine (6 μg/ml), and brefeldin A (10 μg/ml). The cells were then washed with serum-free RPMI 1640 medium and incubated in the presence and in the absence of SCNEE (300 μM) for 30 min at 37 °C. At the end of incubation, cells were washed with PBS, and the activity of APLT was determined as described above.

Two-dimensional High Performance Thin Layer Chromatography (HPTLC)—HL-60 cells with integrated NBD-PS (1.7 nmol × 10<sup>6</sup> cells/ml for 10 min at 4 °C) were incubated at 28 °C for 20 min. At the end of incubation, lipids were extracted using the Folch procedure (39) and separated by two-dimensional HPTLC on Silica G plates (Whatman and Schleicher & Schuell). The plates were first developed with a solvent system consisting of chloroform, methanol, 28% ammonium hydroxide (65:25:5, v/v/v). After drying the plates with a forced air blower to remove the solvent, the plates were developed in the second dimension with a solvent system consisting of chloroform:acetone:methanol:glacial acetic acid:water (50:20:10:10:5, v/v/v). NBD-PS was localized by exposure of HPTLC plates to UV light by using a Fluor-S™ Multimager (Bio-Rad). The phospholipids were visualized by exposure to iodine vapors. The identified spots were scraped, and the silica was transferred to tubes. Lipid phosphorus was determined by the submicro-method as described by Bottcher et al. (40). In addition, TLC plates were scanned, and fluorescence of NBD-PS on plates was estimated using Fluor-S™ Multimager.

PS Exposure on Cell Surface—PS exposure on the surface of cells was determined by flow cytometric detection of annexin V staining using a protocol outlined in the annexin V-fluorescein isothiocyanate apoptosis detection kit (BioVision Research Products, Mountain View, CA). Briefly, HL-60 cells (0.5 × 10<sup>6</sup>) exposed to either SNCEE (50–300 μM) or GSNO (50–300 μM) for 30 min at 37 °C in serum-free RPMI 1640 medium without phenol red, washed once with PBS, and resuspended in binding buffer were stained with annexin V (0.5 μg/ml) and propidium iodide (0.6 μg/ml) for 5 min at room temperature. After staining, cells were immediately analyzed using a FACSscan flow cytometer (BD Biosciences) with simultaneous monitoring of green fluorescence (530 nm, 30 nm bandpass filter) for annexin V-fluorescein isothiocyanate and red fluorescence (long pass emission filter that transmits light >650 nm) associated with propidium iodide. Ten thousand events were collected and analyzed using the LYSIS™ II software (BD Biosciences).

Caspase-3/7 Activity Assay—Caspase-3/7 activity was measured using a luminescence Caspase-Glo® 3/7 assay kit (Promega, Madison, WI). HL-60 cells (4 × 10<sup>5</sup>) were incubated in serum-free RPMI 1640 medium without phenol red in the presence of SNCEE (300 μM) or GSNO (300 μM) for 30 min at 37 °C. At the end of the incubation, cells were washed twice, and caspase-3/7 activity was measured. Luminescence was measured using a plate reading chemiluminometer ML1000 (Dynatech Laboratories). Activity of caspase-3/7 was expressed as luminescence arbitrary units per mg of protein.

Nuclear Fragmentation—Nuclear condensation/fragmentation of Hoechst 33342 (1 μg/ml)-labeled cells was evaluated as described previously (22). Cells were scored using a fluorescence microscope (Nikon ECLIPSE TE 200, Tokyo, Japan) equipped with a digital Hamamatsu CCD camera (C4742-95-12NBR).

Phagocytosis of HL-60 Cells by RAW 264.7 Macrophages—RAW 264.7 macrophages were seeded into an 8-well chamber slide (5 × 10<sup>4</sup> cells/well) and cultured overnight in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Naïve or nitrosatively modified HL-60 cells (i.e. treated in the presence of SNCEE (50–300 μM) as described above) were loaded with Cell Tracker Orange™ (10 μM) for 10 min at 37 °C. Cells were then washed twice and co-cultured with macrophages for 1 h at 37 °C. To inhibit caspase activation, HL-60 cells were pretreated with a pan-caspase inhibitor, Z-VAD-fmk (10 μM, for 30 min at 37 °C). To prevent oxidative damage leading to nonspecific changes in membrane biophysical properties, HL-60 cells pretreated with Z-VAD-fmk were exposed to a potent lipid antioxidant, etoposide (50 μM, for 1 h at 37 °C). In the same experiments macrophages were stimulated by lipopolysaccharide (LPS, 0.1 μg/ml for 6 h at 37 °C) and zymosan (0.25 mg/ml for 1 h at 37 °C). After incubation, unbound target cells were washed three times with RPMI 1640 medium and three times with PBS and fixed with solution of 2% formaldehyde in PBS containing Hoechst 33342 (1 μg/ml) for 1 h at 4 °C. 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 Metal Imaging Series™ software version 4.6 (Universal Imaging Corp., Downingtown, PA). A minimum of 300 macrophages was analyzed per experimental condition. Results are expressed as the percentage of phagocytosis-positive macrophages.
Superoxide Production—Intracellular production of $O_2^-$ was assessed using the dihydroethidium (DHE) oxidation assay (41). DHE has been specifically recommended for assessments of superoxide production in cells because of its relatively high specificity (41). Macrophages ($0.3 \times 10^6$/well) were preincubated with DHE-DA (10 $\mu$M for 10 min at 37 °C) and then stimulated by zymosan (0.25 mg/ml) for 1 h at 37 °C. At the end of incubation, cells were washed three times with PBS, fixed with a solution of 2% formaldehyde in PBS. Treated cells were then examined under a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu charge-coupled device camera (C4742-95-12NBR) and analyzed using the MetaImaging Series™ software version 4.6 (Universal Imaging Corp.).

Nitric Oxide Production—Intracellular production of NO was assessed using the DAF-2DA oxidation assay (42). DAF-2DA has been extensively used for analysis of NO production in cells. In contrast to other NO scavengers, DAF-2 does not readily react with NO metabolites (NO$_3^-$ and NO$_2^-$) and reactive nitrogen and oxygen species (O$_2^-$, H$_2$O$_2$, or ONOO$^-$) (34). Acetylated DAF-2DA readily penetrates into cells and, after hydrolysis, acts as a sensitive and one of relatively specific fluorogenic reagents for NO. Naïve and LPS-stimulated (0.1 $\mu$g/ml, for 6 h at 37 °C) macrophages ($0.3 \times 10^6$/well) were incubated with DAF-2DA (2 $\mu$M, for 1 h at 37 °C) and were then incubated in the presence of zymosan (0.25 mg/ml) for 1 h at 37 °C. At the end of incubation, cells were washed three times with PBS and fixed with a solution of 2% formaldehyde in PBS. Treated cells were then examined under a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu charge-coupled device camera (C4742-95-12NBR) and analyzed using the MetaImaging Series™ software version 4.6 (Universal Imaging Corp.).

Statistics—The results are presented as either mean ± S.E. or mean ± S.D. values from at least three experiments, and statistical analyses were performed by either paired/unpaired Student’s $t$ test or one-way ANOVA. The statistical significance of differences was set at $p < 0.05$.

RESULTS

Exposure of Cells to SNCEE but Not GSNO Results in Elevation of S-NO Thiols and Depletion of Reduced Thiols—In our initial studies, we employed two physiologically relevant trans-nitrosylating reagents, SNCEE (33, 43) and GSNO (44). The former readily enters cells and hence is expected to cause trans-nitrosylation of proteins within intracellular environments; the latter is poorly cell-permeable and therefore can trans-nitrosylate proteins in extracellular compartments.

UV-induced decomposition of S-nitrosothiols quantitatively releases NO$^+$ (35). Therefore, we utilized UV exposure and DAF-2 as a specific probe for released NO$^+$ to assess S-nitrosylation in homogenates of HL-60 cells. We found that the endogenous level of S-nitrosothiols in HL-60 cells was low and did not exceed 50 pmol/mg protein, in line with data reported previously (43, 45). Incubation of HL-60 cells with SNCEE (50–300 $\mu$M) for 30 min at 37 °C resulted in a robust and dose-dependent accumulation of intracellular S-nitrosothiols (Fig. 1A). A 7-fold increase in the S-nitrosothiol content (to 349 ± 20 pmol/mg protein) was detected after exposure of cells to 300 $\mu$M SNCEE (Fig. 1A). In contrast, HL-60 cells exposed to GSNO revealed no significant accumulation of S-nitrosothiols as compared with control untreated cells (Fig. 1A).

$S$-trans-Nitrosylation converts –SH cysteines in S-NO derivatives (27, 28). Thus, a decrease in thiol content is expected as a result of S-nitrosylation. Indeed, we found that SNCEE at a concentration of 300 $\mu$M was able to significantly deplete low molecular weight thiols (GSH) (Fig. 1B, panel a) and protein –SH (panel b) in HL-60 cells exposed to SNCEE or GSNO. HL-60 cells were incubated in the presence of absence of SNCEE and GSNO in serum-free RPMI 1640 medium without phenol red for 30 min at 37 °C. At the end of incubation, cells were washed with PBS, and reduced thiols and intracellular S-nitrosothiols were measured as described under "Experimental Procedures." (*, $p < 0.05$ versus untreated cells, mean ± S.E., ANOVA, n = 5.)

FIGURE 1. Effect of SNCEE and GSNO exposure on the contents of reduced low molecular weight thiols, protein –SH groups, and S-nitrosothiols in HL-60 cells. A, accumulation of S-nitrosothiols in HL-60 cells after treatment with SNCEE or GSNO. B, depletion of low molecular weight (panel a) and protein –SH (panel b) in HL-60 cells exposed to SNCEE or GSNO. HL-60 cells were incubated in the presence or absence of SNCEE and GSNO in serum-free RPMI 1640 medium without phenol red for 30 min at 37 °C. At the end of incubation, cells were washed with PBS, and reduced thiols and intracellular S-nitrosothiols were measured as described under "Experimental Procedures." (*, $p < 0.05$ versus untreated cells, mean ± S.E., ANOVA, n = 5.)
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**FIGURE 2. Activity of APLT in cells subjected to nitrosative stress.** HL-60 cells were incubated in the presence or absence of SNCEE and GSNO in serum-free RPMI 1640 medium without phenol red for 30 min at 37 °C. At the end of incubation, activity of APLT was measured using fluorescently labeled NBD-PS as described under “Experimental Procedures.” (*, p < 0.05, SNCEE versus untreated and GSNO-exposed cells, mean ± S.E., ANOVA, n = 7). Note that cell-permeable SNCEE caused decreased APLT activity, but cell-impermeable GSNO did not.

(The 2). The estimated amounts of NBD-PS that were taken up by HL-60 cells over 20 min of incubation are about 65%; this corresponds to the rate of uptake about 40 pmol/min/10⁶ cells. These numbers are in line with the reported estimates for PS internalization (20, 46–49). Most importantly, after treatment with SNCEE (300 μM), the amounts of internalized NBD-PS and the rate of internalization were reduced to 43% of their values in the absence of SNCEE. In contrast, HL-60 cells treated with GSNO revealed no significant changes in APLT activity (Fig. 2).

To determine whether NBD-PS remains intact after its incorporation and uptake, we measured the contents of PS in control cells and cells with incorporated NBD-PS. To this end, we integrated NBD-PS into HL-60 cells under the same conditions as those used for measurements of APLT activity. At the end of the incubation, lipids were extracted and resolved by two-dimensional HPTLC. We found that NBD-PS migrated differently than PS. The corresponding spots of phospholipids were scraped, and lipid phosphorus was determined. When HL-60 cells were incubated in the presence of NBD-PS (1.7 nmol/10⁶ cells, 10 min at 4 °C), up to 0.61 ± 0.08 nmol/10⁶ cells of NBD-PS were integrated into the cells. No changes in the content of fluorescently labeled PS were found after incubation of HL-60 cells for 20 min at 28 °C (the incubation time used in all experiments in which assessments of APLT internalization were performed). We estimated that after the incubation, the amount of NBD-PS was 0.64 ± 0.19 nmol/10⁶ cells. We additionally assessed the amounts of NBD-PS in cells by measuring its fluorescence after HPTLC separation. HPTLC plates were scanned, and the fluorescence from NBD-PS spots was quantified using Fluor-S™ Multimager (Bio-Rad). Similarly, no changes in the amounts of NBD-PS in HL-60 cells were found after 20 min of incubation.

In addition to inhibition of APLT, activation of scramblase, an enzyme that translocates phospholipids both in- and outward, may contribute to externalization of PS in HL-60 cells treated with SNCEE. To test this, we performed measurements of scramblase activity using fluorescently labeled PC, NBD-PC as a substrate. In contrast to NBD-PS, ~90% of exogenously added NBD-PC remained on the surface of HL-60 cells over a 20-min incubation period. As expected, the addition of Ca²⁺ to the incubation medium did not cause a significant increase in the scramblase activity such that 85 ± 5% of total NBD-PC was still localized on the cell surface. However, treatment of HL-60 cells with SNCEE caused translocation of NBD-PC from the outer to the inner leaflet of plasma membrane such that ~70 ± 5% of the total amount of NBD-PC was localized in the outer leaflet. An additional 7% of NBD-PC was internalized when Ca²⁺ was added to the incubation medium. Thus, the contribution of Ca²⁺ in the activation of scramblase activity in HL-60 cells was relatively small. The calculated maximal rates of NBD-PC internalization (during initial 2 min of incubation) were 18.9 ± 0.3 and 27.7 ± 0.8 pmol/min/10⁶ cells in the absence and in the presence of Ca²⁺, respectively. This is significantly lower than the maximal rate of NBD-PS internalization (40 pmol/min/10⁶ cells). Given that scramblase translocates phospholipids randomly (74, 75) and that normally the amount of PS in plasma membrane of HL-60 cells does not exceed 8.5% of total phospholipids resulting in the PC:PS ratio of 5.1 (79), the physiological rate of PS scrambling should be an order of magnitude lower than the rate of APLT-dependent translocation. Furthermore, because most of PS is confined to the inner leaflet of the plasma membrane, its internalization in normal HL-60 cells is predominantly and specifically dependent on APLT activity. However, after exposure to SNCEE (and likely during apoptosis), not only inhibition of APLT activity but also a 3-fold activation of scramblase may play a significant role in PS externalization.

To determine the extent to which the uptake of NBD-PS was selective for PS and mediated by the APLT, rather than by non-specific endocytosis, we measured APLT activity in cells pre-treated with a mixture of endocytosis inhibitors that included nystatin (25 μg/ml), genistein (200 μM), chlorpromazine (6 μg/ml) and brefeldin A (10 μg/ml). We found no differences in the APLT activity between control cells and cells treated with the inhibitors of endocytosis (96 ± 4% of the control value after treatments with the inhibitors). In addition, the decrease of APLT activity induced by SCNCEE was also unaffected by the inhibitors (45 ± 2% of the control value after treatments with inhibitors versus 43 ± 3% without inhibitors).

**SNCEE Triggers PS Externalization without Activating the Common Apoptotic Pathway**—Furthermore, we were interested in determining the extent to which S-nitrosylation of cellular proteins and subsequent inhibition of APLT is associated with changes in PS externalization in HL-60 cells. To this end, cells were exposed to SNCEE (300 μM) for 30 min and subsequently co-stained with Hoechst 33342 (blue) and annexin V (green) for visualization of nuclear condensation and PS exposure, respectively. We found that cells readily externalized PS on their surface in response to SNCEE as evidenced by the appearance of typical patched fluorescent spots of annexin V (Fig. 3A). No PS was detectable on the surface of nontreated cells (Fig. 3A). To quantify the number of cells with externalized PS, we used flow cytometric analysis. Less than 4.8 ± 0.1% of control cells exposed PS on their surface. SNCEE caused a dose-
In addition, no nuclear condensation or fragmentation was observed when HL-60 cells were exposed to SCNEE (Fig. 3A). During apoptosis, PS externalization usually parallels caspase-3 activation (50). However, no activation of caspase 3/7 was found in either SNCEE-treated cells (300 μM, 30 min) or in cells incubated for 0.5, 2, 4, 6, and 18 h after the SNCEE treatment (Fig. 3C). Thus, at the time when SNCEE-induced externalization of PS was associated with phagocytosis of target cells, the caspase-dependent pathways of apoptosis were not initiated. This is in line with the reported data indicating that in normal non-apoptotic cells caspase 3 is S-nitrosylated and that denitrosylation of caspase 3 is required for its catalytic activation (51). Therefore, SNCEE-induced PS externalization was disconnected from the common caspase-dependent pathway of apoptosis. No significant increase in the number of annexin V-positive HL-60 cells (versus nontreated controls) was observed after treatment with GSNO (Fig. 3B).

**Dithiothreitol (DTT) Reverses SNCEE-induced APLT Inactivation and PS Externalization in HL-60 Cells**—Recently, we have shown that vicinal di-thiols such as thioredoxin, dihydroliopic acid, and DTT, in addition to their well known ability to reduce disulfides, can also de-nitrosylate S-nitrosylated proteins in live cells (43). Thus, we hypothesized that inhibition of APLT activity and PS externalization on the cell surface induced by nitrosative stress can be reversed by DTT. Measurements of S-nitrosothiols revealed that ~65% of intracellular thiols were de-nitrosylated when DTT was added after SNCEE treatment (Fig. 4C). Importantly, DTT was able to reverse SNCEE-induced inhibition of APLT up to 91% of its rate characteristic of control HL-60 cells (the rate of NBD-PS internalization was as high as 38.8 ± 1.5 pmol/min, insignificantly different from control HL-60 cells) (Fig. 4A). DTT was also able to reverse SNCEE-induced PS externalization (Fig. 4B). These results demonstrate that SNCEE-induced nitrosative/oxidative stress uncoupled from apoptotic caspase activation was responsible for the inactivation of APLT and externalization of PS in HL-60 cells.

**RAW 264.7 Macrophages Elicit Enhanced Phagocytic Activity toward “Nitrosylated” HL-60 Cells**—Because externalized PS is an important and universal recognition signal for engulfment of apoptotic cells by macrophages, we further tested whether exposure of non-apoptotic HL-60 cells to nitrosative stress would be sufficient for their phagocytosis. HL-60 cells pre-labeled with Cell Tracker Orange were treated with SNCEE (300 μM) and then co-incubated with RAW 264.7 macrophages. Relatively low levels of phagocytosis-positive macrophages (less than 2.0%) were detected when control (nontreated) HL-60 cells were co-incubated with RAW 264.7 macrophages (Fig. 5A). In contrast, SNCEE-treated HL-60 cells were readily engulfed by RAW 264.7 macrophages (Fig. 5A). The number of positive macrophages was dependent on the concentration of SNCEE and increased up to 15% after their co-incubation with HL-60 cells exposed to 300 μM of SNCEE (Fig. 5B). To exclude potential minimal activation of caspases, HL-60 cells were pre-treated with the pan-caspase inhibitor Z-VAD-fmk. We found that SNCEE caused a quantitatively similar pattern of engulfment by macrophages both in the absence and in the presence of Z-VAD-fmk (Fig. 5B). To further test whether oxidative
damage potentially leading to nonspecific changes in membrane biophysical properties may be involved in recognition of nitrosylated cells by macrophages, we used etoposide, a previously reported potent inhibitor of lipid peroxidation in HL-60 cells (18, 52), along with Z-VAD-fmk. We found that etoposide/Z-VAD-fmk-pretreated cells incubated with SNCEE were effectively recognized and engulfed by RAW 264.7 macrophages (Fig. 5B).

Activation of macrophages is associated with the generation of reactive oxygen species and reactive nitrogen species known to cause both S-nitrosylation and oxidation of low molecular weight thiols and protein cysteines (31). Therefore, we were anxious to further test our hypothesis in a physiologically more relevant environment by using activated RAW 264.7 macrophages and HL-60 cells as a source of nitrosative stress and target cells, respectively. First, we confirmed that LPS-stimulated and zymosan-treated RAW 264.7 macrophages indeed generate \( \text{O}_2^- \) and \( \text{NO}^-/\text{H}_2\text{O}_2 \). By using DHE-DA and DAF-2DA as specific probes for \( \text{O}_2^- \) and \( \text{NO}^-/\text{H}_2\text{O}_2 \), respectively (41, 42), we found that generation of \( \text{NO}^-/\text{H}_2\text{O}_2 \) in LPS/zymosan-stimulated DAF-2DA-loaded RAW 264.7 macrophages was significantly increased (Fig. 6A). Similarly, zymosan caused a robust intracellular production of \( \text{O}_2^- \) radicals as evidenced by the increased number of DHE-positive cells (Fig. 6B). Furthermore, naïve HL-60 cells co-incubated with LPS-stimulated macrophages in the presence of zymosan for 1 h at 37 °C were readily engulfed (Fig. 7A). The number of positive macrophages was increased to 26.4 \( \pm \) 1.7% (versus 2% in control with nonstimulated macrophages without zymosan). The number of phagocytosis-positive macrophages was significantly decreased when annexin V was added to the incubation medium confirming the participation of externalized PS as an essential recognition signal (Fig. 7B).

Moreover, DAF-2, a non-cell-permeable scavenger of \( \text{NO}^-/\text{H}_2\text{O}_2 \), added to the incubation system was able to quench \( \text{NO}^- \) pro-
Nitrosative Stress Regulates Cell Clearance

Nitrosative Stress Drives PS-dependent Clearance of Target Cells—This work demonstrates for the first time that nitrosative stress causes inhibition of APLT, externalization of PS on the cell surface, and subsequent elimination of these cells by macrophages. The physiological significance of these findings is underscored by the importance of effective apoptotic cell clearance, whose success provides for safe elimination of unwanted cells without accompanying excessive inflammation and/or autoimmunity. Thus, paradoxically, NO\textsuperscript{*} contributes to efficient phagocytosis of target cells.

**DISCUSSION**

Effect of Nitrosative Stress Induced by Stimulated RAW 264.7 Macrophages on the Phagocytosis of HL-60 Cells—RAW 264.7 macrophages were stimulated with LPS and/or zymosan for 6 h at 37 °C. A, typical microphotographs of engulfment of HL-60 cells by RAW 264.7 macrophages (arrows, upper right panel). In the presence of annexin V (AnV, lower left panel) or DAF-2 (lower right panel) phagocytosis of HL-60 cells by RAW 264.7 macrophages is decreased. B, effect of annexin V and DAF-2 on number of phagocytosis-positive HL-60 cells by RAW 264.7 macrophages (*, p < 0.05, LPS + zymosan versus control; #, p < 0.05, LPS + zymosan + annexin V and versus LPS + zymosan + DAF-2 versus LPS + zymosan; mean ± S.E., ANOVA, n = 5). HL-60 cells were labeled with Cell Tracker Orange\textsuperscript{TM} (10 μM for 10 min at 37 °C). Then cells were co-incubated with LPS-stimulated RAW 264.7 macrophages in the presence of zymosan for 1 h and subsequently analyzed for phagocytosis as described under “Experimental Procedures.”

Although etoposide acts as a pro-apoptotic agent in HL-60 cells after 4–8 h of exposure, during the initial 0.5–1-h period, no pro-apoptotic manifestations (cytochrome c release, caspase activation, and PS externalization) are detectable (18, 52). Most importantly, etoposide completely prevents oxidative membrane modifications. This study shows that nitrosative stress can enhance macrophage engulfment in the absence of other indices of apoptosis. These findings are in line with our previous studies showing that oxidative stress inactivates caspases in human neutrophils, yet induces the simultaneous caspase-independent PS externalization (9). Because the engulfment and removal of dying cells define the “meaning” of cell death (10), one may argue that nitrosative/oxidative stress is ultimately a pro-death event or, at any rate, a pro-clearance event, which could have important implications for the resolution of inflammation.
Nitrosative Stress Regulates Cell Clearance

Role of trans-Nitrosoy and/or Peroxynitrite-dependent Nitrosylation—Nitrosative stress generated by macrophages is an essential step to control microbial pathogens and initiate immune responses (56). At sites of inflammation, where the concentrations of both $\mathbf{O}_2^*$ and NO$^-$ are elevated (29), a highly reactive intermediate, ONOO$^-$, is formed (30, 31). The half-life of ONOO$^-$ in the extracellular environment has been estimated to be on the order of 10 ms, and both ONOO$^-$ and peroxynitric acid (ONOOCOH) can diffuse across biomembranes (30) and modify proteins through S-nitrosylation (32). In addition to direct nitrosylation of protein cysteines, ONOO$^-$ can also nitrosylate highly abundant intracellular low molecular weight thiols to yield S-nitrosothiols; the latter can then trans-nitrosylate protein cysteines, including those of APLT, in cells (27). Accumulation of low molecular weight S-nitrosothiols has been detected in cerebrospinal fluid, plasma, and synovial fluid of patients with a variety of inflammation-associated diseases (57–60). However, as our data with GSNO demonstrate, these extracellular S-nitrosothiols are not likely candidates as effective regulators of PS externalization and phagocytosis.

S-Nitrosylation and Oxidation of APLT Cysteines—Our assessments of S-nitrosylated thiols accumulated in HL-60 cells as compared with the loss of –SH groups in the trans-nitrosylation suggests that S-nitrosylation represents only a fraction of oxidatively/nitrosatively modified proteins. We found significant oxidation of protein –SH groups as well as low molecular weight thiols in cells exposed to SNCEE, which can be related to both homolytic decomposition of nitrosothiols and modification of sulfhydryls to disulfides by reactive species derived from NO$^-$ (30, 61). It is possible that both S-nitrosylation and oxidation of cysteine residues were responsible for the loss of APLT activity. The inhibition of APLT as well as PS externalization in cells exposed to nitrosative stress were reversible by a strong reducing agent, DTT. Moreover, this was accompanied by a significant de-nitrosylation of intracellular S-nitrosothiols. Recently, we have reported that several vicinal thiols such as thioredoxin, dihydrodialdoic acid, and DTT are catalytically active in de-nitrosylating S-nitrosylated proteins in model systems and cells (43). In line with the previously published finding that S-nitrosylation is a reversible process (62, 63), our results indicate that DTT-driven de-nitrosylation might contribute to the reconstituted activity of the APLT enzyme. However, DTT is also a potent reductant of disulfide bonds (23). Therefore, direct reduction of S–S bonds as a potential mechanism of the APLT activity recovery cannot be excluded. Nonetheless, in our experiments with LPS-stimulated macrophages, phagocytosis of target cells was significantly suppressed by the NO$^-$ scavenger DAF-2 (34). This suggests that whether nitrosylation or oxidation is the major contributor to mechanism(s) of APLT modification, NO$^-$ plays a prominent role in the execution of cell clearance.

Access of APLT Cysteine Residues to Nitrosylation—A number of proteins have been identified as targets for S-nitrosylation altering their function (28). For example, S-nitrosylation of phosphatases, protein kinases, caspases, and tissue transglutaminases has been associated with the loss of catalytic activity (28, 64). APLT is a lipid-selective ATPase involved in transport of aminophospholipids (15), mainly PS, from the outer to the inner leaflet of plasma membrane (15, 16). As an integral membrane protein (15, 16) containing cysteine residues (65, 66), APLT is expected to be a potential target for S-nitrosylation. It has been shown that the enzyme is sensitive to oxidation/alkylation of its –SH groups (17, 26). In addition, several reports demonstrated that a cysteine residue is critical for activity or conformational integrity of APLT (23). According to our proposed mechanism, activated macrophages cause APLT nitrosylation and PS externalization indiscriminately in many bystander cells. This is in line with the data of Brown and Savill (67) who have demonstrated that activated macrophages induced PS externalization in bystander leukocytes. Thus, any cell in the vicinity of activated macrophages may become a target for phagocytosis. However, not only reactive oxygen and nitrogen species (68, 69), but other factors as well, including cytokines (70) and growth factors (71), contribute to responses of bystander cells to activated macrophages. Our measurements of APLT activity revealed a strong inhibitory effect of SNCEE and inability of GSNO to inactivate the enzyme. Consequently, PS was externalized and phagocytosis was stimulated only in response to SNCEE but not to GSNO. Because GSNO does not readily permeate cells, we conclude that functionally critical sulfhydryl groups in the APLT protein are located within the cytosolic domain of the enzyme or are buried in a hydrophobic environment unavailable for GSNO (72). This is also supported by other reports on the inhibitory effects of sulfhydryl oxidation (by diamide) or cross-linking (by pyridylthioethyamine) of the translocase activity (23). Moreover, successful attempts to reverse the inhibition by DTT, but not by the membrane-impermeable reducing agent GSH, indicate that at least one critical APLT cysteine is embedded in the membrane or exposed to the cytoplasmic surface (23).

Although APLT selectively translocates aminophospholipids (particularly PS) from the outer to the inner leaflet of plasma membrane (73), “scramblase” activity randomly transports different phospholipids, including PS, in both directions. Thus both inhibition of APLT and activation of scramblase can contribute to the PS externalization. The scrambling of phospholipids is dependent on transbilayer phospholipid gradient and activated by $\mathbf{Ca}^{2+}$ (74, 75). It has been shown that phospholipid scrambling is enhanced by modification of one or more –SH groups (76, 77) and is suppressed by the reducing agent DTT (78). Thus it is possible that SNCEE-induced S-nitrosylation of the scramblase sulfhydryl groups contributed to the activation of scramblase and PS externalization.

Although inhibition of APLT is believed to be pivotal for the externalization of PS during apoptosis, the role and contribution of scramblase 1 (PLSCR1) in disruption of PS asymmetry across the plasma membrane are more debatable (74, 80–82). Several reports provide evidence that scramblase activation alone is not sufficient to cause PS externalization (19, 24, 83, 84). Moreover, it has been demonstrated that expression of PLSCR1 is neither necessary nor sufficient for PS exposure during Fas-triggered apoptosis (19). In addition, no correlation between the level of expression of the phospholipid scramblase and the capacity of these cells to externalize PS during apoptosis was found (24, 83). Finally, blood platelets from PLSCR1$^{-/-}$ mice showed normal capacity to expose PS upon cell activation.
However, results from different laboratories demonstrate the importance of concomitant inhibition of APLT and activation of scramblase for PS exposure (75, 85–87). In line with this view, our data suggest that SNCEE-induced S-nitrosylation and subsequent PS exposure on the surface of HL-60 cells is dependent on both APLT inactivation and scramblase activation, whereby the contribution of the former is likely to be more significant.

Nitrosative Stress Regulates Cell Clearance

Nitrosative Stress as a Regulator of the Inflammatory Response and Cytokine Production—Disruption of apoptotic cell removal may yield pro-inflammatory conditions (6) that are associated with a number of autoimmune and chronic inflammatory diseases (3, 7–9). Moreover, PS-dependent clearance of apoptotic cells by macrophages plays an active role in the resolution of inflammation, through production of anti-inflammatory cytokines such as transforming growth factor-β and down-regulation of pro-inflammatory mediators such as tumor necrosis factor-α (1, 2). In particular, failure to clear neutrophils undergoing apoptosis resulting in their accumulation is a hallmark of chronic inflammation accompanied by a persistent production of pro-inflammatory cytokines (88, 89).

We and others have demonstrated that the PS signaling pathway turns off the production of pro-inflammatory cytokines and reactive oxygen and nitrogen species by activated macrophages (2, 5). Thus, nitrosative stress realized via NO-dependent macrophage processes and causing PS externalization in target cells acts as a novel mechanism for regulation of the inflammatory response. In line with this, therapy of inflammatory conditions using NO-releasing drugs has shown considerable promise. Indeed, addition of an NO moiety to nonsteroidal anti-inflammatory drugs sufficiently reduced their toxicity and enhanced their therapeutic effects (90, 91). Nanoparticles, especially SWCNT, are widely utilized for improvements of drug delivery and therapeutic efficacy of drugs (92). Nonfunctionalized SWCNT, however, are poorly recognized by macrophages. Our recent studies showed that coating of SWCNT with PS resulted in a remarkable improvement in their recognition, binding, and clearance of PS-positive cells by macrophages trigger the resolution of inflammation through production of anti-inflammatory cytokines and down-regulation of pro-inflammatory mediators (99).
target cells by inhibiting APLT activity and promotes the disposal of these cells by macrophages. Thus, nitrosative/oxidative stress may significantly contribute to the clearance of effete cells and, hence, to the resolution of inflammation (Fig. 8). These findings suggest that nitric oxide produced by activated macrophages, through its effects on PS-dependent phagocytosis of neighboring target cells, is engaged in a feedback mechanism limiting excessive inflammatory responses. Targeting of these events could ultimately provide novel avenues for therapeutic intervention in chronic inflammatory diseases.

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