Defects in intracellular oxidative metabolism of neutrophils undergoing apoptosis

P. K. Narayanan, K. Ragheb, G. Lawler, and J. P. Robinson

Cytometry Laboratories, Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

Abstract: Apoptosis permits neutrophil recognition by macrophages, thereby not only limiting potential cytotoxicity but also promoting resolution of inflammation. A direct relationship between apoptosis and intracellular hydrogen peroxide (H_2O_2) production was observed in phorbol 12-myristate 13-acetate (PMA) -stimulated neutrophils aged in culture. A significant decrease in intracellular H_2O_2 production was observed in aging neutrophils at 12, 24, and 48 h. However, intracellular superoxide anion production in response to PMA stimulation was preserved up to 24 h, implying retention of intracellular signaling pathways leading to NADPH oxidase stimulation. A significant decrease in the cytoplasmic content and activity of Cu,Zn superoxide dismutase was responsible for the observed decline in intracellular H_2O_2 production in apoptotic neutrophils. Intracellular glutathione content also decreased concomitantly with H_2O_2 production. These observations indicate that onset of apoptosis in neutrophils is in part mediated by oxidative stress resulting from the down-regulation of key antioxidant defense systems of the cell, namely superoxide dismutase and glutathione. *J. Leukoc. Biol.* 61: 481–488; 1997.

Key Words: *human · free radicals · FACS*

INTRODUCTION

Neutrophils play an important role in mediating tissue injury in a wide range of inflammatory diseases [1]. Events leading to tissue injury are often initiated by the release of reactive oxygen species (ROS), lysosomal enzymes, and other proteins that act as mediators of inflammation from activated neutrophils [2–4]. Neutrophils direct these mediators to intercept and kill microbes or other external stimuli that gain access across the body's physical barrier [5]. When these defense mechanisms are directed inappropriately, tissue damage occurs [1] and it eventually becomes necessary to remove neutrophils to limit such potential cytotoxicity and for normal resolution to occur [6]. A major route of disposal of extravasated neutrophils has been postulated to be via apoptosis [7–9]. Neutrophils undergoing apoptosis express surface antigens that are recognized by vitronectin receptors on macrophages [10, 11], which are responsible for the ultimate removal of the neutrophil.

The concept that aging neutrophils undergo apoptosis evolved recently [12]. Neutrophils undergo apoptosis in a manner similar to that observed in many other cell types: with condensation of nuclear chromatin, compaction of cytoplasmic organelles, cell shrinkage, and changes at the cell surface [13, 14]. Human neutrophils shed surface FcγRIIIb(CD16) and acquire annexin V binding sites during apoptosis in vitro [15, 16]. These changes take place without loss of membrane integrity, as shown by propidium iodide (PI) exclusion [14, 17]. Endogenous endonuclease activity, which is shown by in situ labeling of DNA strand breaks with biotinylated dUTP utilizing exogenous terminal deoxyribonucleotide transferase, is also characteristic of apoptosis [18, 19]. Apoptosis then allows recognition and ingestion of apoptotic neutrophils by macrophages and fibroblasts and represents a mechanism by which neutrophils are cleared from the inflammatory site [7, 8, 10, 11, 20, 21].

Current understanding of oxidative function in apoptotic neutrophils is based primarily on the measurement of extracellular products [22]. Previous findings have suggested that extracellular superoxide anion (O_2^-) generation in response to a receptor-independent stimulus such as phorbol 12-myristate 13-acetate (PMA) was preserved in apoptotic neutrophils [22]. According to our initial findings, intracellular O_2^- anion formation in PMA-stimulated neutrophils aged in vitro was maintained or increased while intracellular hydrogen peroxide (H_2O_2) production detected concomitantly decreased significantly. Diminished production

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SOD, superoxide dismutase; GSH, glutathione; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; mBrB, monobromobimane; HE, hydroethidine; DMSO, dimethyl sulfoxide; NEM, *N*-ethylmaleimide; DPI, diphenylene iodonium; HE, hydroethidine; EB, ethidium bromide; DCF, 2′,7′-dichlorofluorescein; mClB, monochlorobimane; GST, glutathione *S*-transferase; PI, propidium iodide; FALS, forward angle light scattering; HLS, high light-scatter region; LLS, low light scatter; NGF, nerve growth factor.

Correspondence: P. K. Narayanan, M888, Life Sciences Division, Los Alamos National Laboratory, Los Alamos, NM 87545.

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of H_2O_2 could be secondary to either decreased functional activity of superoxide dismutase (SOD) or increased availability of the tri-peptide glutathione (GSH). We therefore investigated the possibility of modulation of SOD activity and GSH content in vitro during the course of the onset of apoptosis in human neutrophils.

MATERIALS AND METHODS

Buffers and reagents

Hanks' balanced salt solution (HBSS) containing 137 mM NaCl, 5.4 mM KCl, 0.7 mM Na_2HPO_4, 1.2 mM $NaHCO_3$, 12.2 mM glucose (0.22%), 27.5 mM Tris, 1.87 mM Ca^{2+}, 0.8 mM Mg^{2+}, and 0.1% bovine serum albumin (BSA), pH 7.4, was used for incubating neutrophils for subsequent stimulation with PMA. Phosphate-buffered saline (PBS) containing 147 mM NaCl, 0.041 mM $Na_2HPO_4 \cdot 7H_2O$, 2.3 mM Na_2HPO_4, 15 mM $NaNO_3$, 2 mM ethylenediaminetetraacetate (EDTA), 10 mM glucose, pH 7.4 (Difco Laboratories, Detroit, MI) was used for reagent dilutions. Phosphate buffer containing 50 mM KH_2PO_4, 50 mM Na_2HPO_4, and 0.1 mM EDTA, pH 7.8 (solution A) was used for ferricytochrome *c* reduction assay. Xanthine and xanthine oxidase (Sigma Chemical Company, St. Louis, MO) were employed to generate O_2^- in a cell-free system. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) and monobromobimane (mBrB; Molecular Probes, Eugene, OR) dissolved in absolute ethanol were used at a final concentration of 20 and 40 μM, respectively. Hydroethidine (HE; Molecular Probes) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM (final concentration 10 μM). PMA (LC Services Corp., Woburn, MA) was dissolved in DMSO to a 3.25 mM stock solution and used at a final concentration of 10 ng/mL [23]. *N*-ethylmaleimide (NEM, Sigma, St. Louis, MO) was dissolved in PBS and used at a final concentration of 100 μM. Potassium cyanide (KCN, Sigma) and diphenyleneiodonium (DPI, Molecular Probes) were used at final concentrations of 2 mM and 20 μM, respectively.

Collection of neutrophils

Peripheral blood was collected from healthy adult volunteers by standard venipuncture methods in a sterile tube containing preservative-free heparin (informed consent in compliance with the regulations of the Committee on the use of Human Research Subjects at Purdue University was obtained). Preparation of human neutrophils from normal venous blood was performed as previously described [8]. Briefly, fresh venous blood was diluted with PBS (maximum dilution 2:1, buffer-blood) and centrifuged through a density gradient medium (Lymphocyte Separation Medium™, Organon Teknika, Durham, NC) at 400 *g* for 30 min at room temperature. All the regions above the red cell layer were removed. The red cell layer was dextran sedimented at 1 *g* in a 37°C water bath for 40 min after adding 10 mL PBS and 3 mL 3% dextran T-500 (Pharmacia). After incubation the supernatant was removed and centrifuged at 200 *g* for 10 min. The remaining red cells were lysed using 2 mL sterile distilled water for 25 s followed by addition of excess PBS. Cells were washed twice in PBS, counted using a Coulter Counter (Coulter Corp., Hialeah, FL), and the concentration adjusted to 2.0×10^6 cells/mL in HBSS for all assays. The cells collected were at least 97% neutrophils as assessed by microscopic examination of cytospin preparations stained with Wright-Giemsa (Hema-Tek Stain Pak, Miles Inc., Elkhart, IN).

Neutrophil culture

Freshly isolated neutrophils were suspended at a density of 5×10^6/mL in Iscove's modified Dulbecco's medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). Cell suspensions were then incubated at 37°C in a 5% CO_2 atmosphere up to 12, 24, and 48 h.

Detection of ROS

Direct intracellular measurement of O_2^- utilized hydroethidine (HE), the sodium borohydride reduced derivative of ethidium bromide (EB). During the oxidative burst, stimulated neutrophils release O_2^- through membrane-bound NADPH oxidase, resulting in oxidation of HE to EB by O_2^-. Ethidium bromide fluoresces brightly upon DNA intercalation, which can be measured (620 nm) directly by flow cytometry [24]. Intracellular H_2O_2 generation was detected using neutrophils loaded with DCFH-DA [25]. This assay depends on the incorporation of DCFH-DA into the hydrophobic lipid regions of the cell, where the acetate moieties are cleaved by esterases yielding the non-fluorescent molecule 2′,7′-dichlorofluorescin (DCFH). DCFH is trapped due to its polarity within the intracellular granules (myeloperoxidase positive) and the cytoplasm. The oxidative potentials of H_2O_2 together with peroxidases are able to oxidize the trapped DCFH to 2′,7′-dichlorofluorescein (DCF), which is fluorescent in the green area of the spectrum (525 nm) [24]. The cell suspension was pre-incubated for 15 min at 37°C for DCFH-DA and 5 min for HE to incorporate the probes into the cells. PMA (10 ng/mL) stimulation was used for all experiments evaluating the oxidative burst.

Measurement of GSH

GSH content in neutrophils was estimated using mBrB, which combines with GSH nonenzymatically at low concentrations. mBrB is more specific for human cells than monochlorobimane (mClB), which combines enzymatically with GSH by means of glutathione *S*-transferase (GST). The predominant transferase found in human cells, πGST, has both a low affinity for and a low catalytic reactivity with mClB [26]. mBrB reacts specifically with GSH at low concentrations in the first 10 min of reaction time. Neutrophils were stained with 40 μM mBrB [27] for 10 min at room temperature after which the samples were run on the flow cytometer. A replicate sample was depleted of GSH by treatment with 100 μM NEM to give a relative measure of nonspecific binding.

Cell viability

Neutrophils were assessed for viability by use of the well-characterized property of live cells to exclude propidium iodide (PI) [28]. PI (10 μg/mL) was added to 2×10^6 cells/mL at room temperature at the completion of each assay and the results read by flow cytometry 10 min later using excitation of 488 nm and emission at 575 nm.

Flow cytometry

Dual-laser flow cytometry and data collection were performed with the use of an EPICS ELITE flow cytometer (Coulter Cytometry) with instrument standard computer, optics, and electronics. Two argon lasers (Cyonics, Uniphase; Innova 300, Coherent) were used as excitation sources. The first laser was used for the excitation of ROS-specific dyes (HE and DCF) and PI at 488 nm. The ultraviolet line (353 nm) of the second laser was used for excitation of mBrB. Optical filters used included a 408-nm dichroic filter for mBrB, 488-nm dichroic filter for 90°LS, 525-nm band pass for DCF, and 610-nm long pass for EB and PI.

A previously defined assay technique was used to collect kinetic measurements on individual cells [29]. Briefly, samples were activated immediately after being run on the cytometer for the first measurement (zero time); this insured that each sample's zero time measurement was a true zero for that tube. Subsequent tubes were run in the manner previously described [29]. Samples were then repeatedly measured in order at specified time intervals.

Functional inactivation of SOD

Reduction of ferricytochrome c

Superoxide anion production via the xanthine/xanthine (x–xo) oxidase system was determined by monitoring the reduction of cytochrome *c* at 550 nm [30, 31]. Solution A, consisting of 0.76 mg (5 μM) xanthine in 10 mL 0.001 N sodium hydroxide and 24.8 mg (2 μM) cytochrome *c* were mixed with 100 mL 50 mM phosphate buffer, pH 7.8, containing

0.1 mM EDTA. A freshly prepared solution of 0.2 units/mL of xanthine oxidase (solution B) was made up in phosphate buffer. After adding 50 μL of sample (water, SOD standards, or unknowns) to 2.9 mL of solution A in a cuvette, the reaction was started by adding 50 μL of solution B.

SOD will compete for superoxide and decrease the reduction rate of cytochrome *c* [31]. To distinguish between nonspecific oxidoreductase and SOD activities, the ferricytochrome *c* was acetylated [32]. Pure Cu,Zn-SOD (cyanide-inhibitable) was used as a standard for these assays. Catalase (40 units/mL) was included to remove H_2O_2. Formation of H_2O_2 will inhibit Cu,Zn-SOD, followed by a shift in the equilibrium in favor of O_2^- production. Measurements were made on a Lambda3 Series spectrophotometer (Perkin-Elmer Corp.). The reaction mixture was kept at 25°C and the reaction started by addition of xanthine (5 μM). The kinetics of the reaction were recorded for a period of 60 s. The contents of the reference cuvette were the same except for the omission of xanthine oxidase. The rate of reduction of cytochrome *c*, inhibited by SOD, is plotted as the reciprocal absorbance change per minute versus concentration of SOD standards [30]. Neutrophils were sonicated and 50 μL of the lysate were added instead of the standards. The SOD activity expressed as nanograms per 10^7 neutrophils was then extrapolated from the standard curve via regression analysis.

Statistical analysis

Triplicate samples were run on the flow cytometer and 5,000 cells were analyzed from each tube. Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance followed by Tukey's procedure for multiple comparisons. The acceptable level of significance was $P < 0.05$.

RESULTS

Light scatter properties and membrane integrity of aging neutrophils

Distinct, time-dependent changes were observed in human neutrophils isolated from peripheral blood and then aged in culture for up to 48 h. Ultrastructural examination by electron microscopy [33], shedding of surface CD16 [16], and in situ labeling of DNA strand breaks with biotinylated dUTP utilizing exogenous terminal deoxyribonucleotide transferase [19] were the primary assays employed to distinguish neutrophils undergoing apoptosis (data not shown).

As described previously, neutrophils were distinguished by applying flow cytometric measurements of forward angle light scattering (FALS) and orthogonal light scattering (90°LS), which reflected their size and increased granularity [34]. A bitmap was applied around the cell population and designated a high-light-scatter (HLS) region (**Fig. 1A**). One hundred percent of the freshly isolated neutrophils occupied the HLS region. Aging in culture altered the light scatter properties of neutrophils significantly by 12 h. As a result of this, a significant percentage of neutrophils shifted from the HLS region to the region designated low light scatter (LLS) when compared with normal freshly isolated neutrophils (Fig. 1B). Untreated neutrophils at 12 h had 77% cells in the HLS region and 18% in the LLS region. At 24- and 48-h time points the percent of cells in the HLS region dropped to 57 and 15.4%, respectively, followed by an increase in the LLS region to 44.6 and 82.3%. The balance were either clumps or damaged cells.

PI, a DNA-binding dye, was used to differentiate between live and dead cells by the property of dye exclusion

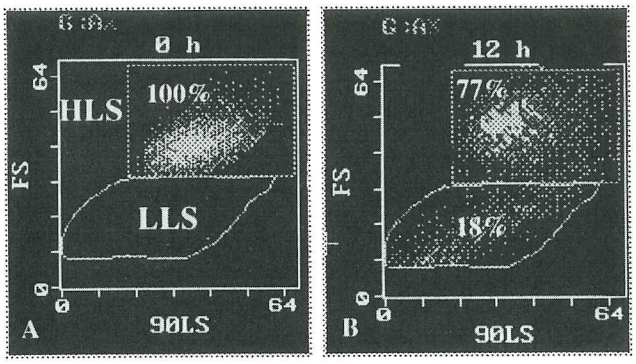

Fig. 1. (A and B) Light scatter of neutrophils at 0 and 12 h. Aging in vitro alters the light scatter properties after which there is a substantial increase in the percentage of cells moving from the HLS region to the LLS region.

by intact plasma membranes. Freshly isolated, untreated neutrophils demonstrated a viability of 99.2 ± 0.14% using this technique. At 12 h the neutrophils could be discriminated into two distinct populations based on their light scatter properties as described earlier. Neutrophils in the HLS region had 99.8 ± 0.07% viability when compared with 42 ± 1.41% viability for cells in LLS region. Viability in the HLS region and the LLS region decreased to 80 ± 3.1 and 20 ± 3.5% at 24 h, and 64 ± 0.2 and 13 ± 0.1% at 48 h.

Intracellular O_2^- and H_2O_2 generation in unstimulated neutrophils

Intracellular O_2^- generation was determined by the increase in EB fluorescence in the presence and absence of 10 ng/mL PMA [24]. Unstimulated neutrophils demonstrated a mean EB fluorescence of 1.65 ± 0.2 [(mean ± SEM) fluorescence units (U)] (**Fig. 2**). EB fluorescence in unstimulated neutrophils at 4 h showed significant alterations when compared with the unstimulated control at zero time where EB fluorescence increased significantly to 3.98 ± 0.03 U without any exogenous stimulation.

As observed earlier with O_2^- production in unstimulated neutrophils, H_2O_2 production also demonstrated changes in unstimulated neutrophils at 4 h. A significant increase in DCF fluorescence was seen in unstimulated neutrophils at 4 h (5.37 ± 0.03 U) when compared with unstimulated neutrophils at 0 time (1.9 ± 0.2 U; Fig. 2). To locate the specific pathway responsible for this increase in H_2O_2 production, neutrophils were incubated with 2 mM cyanide and 20 μM DPI for 10 min at 37°C before estimating H_2O_2 production. Cyanide significantly decreased H_2O_2 production (3.41 ± 0.04 U, $P < 0.001$), whereas DPI did not have any effect on the production of this free radical (**Fig. 3**).

Intracellular O_2^- and H_2O_2 generation in stimulated neutrophils

In freshly isolated PMA-stimulated neutrophils, O_2^- production in terms of mean channel EB fluorescence was

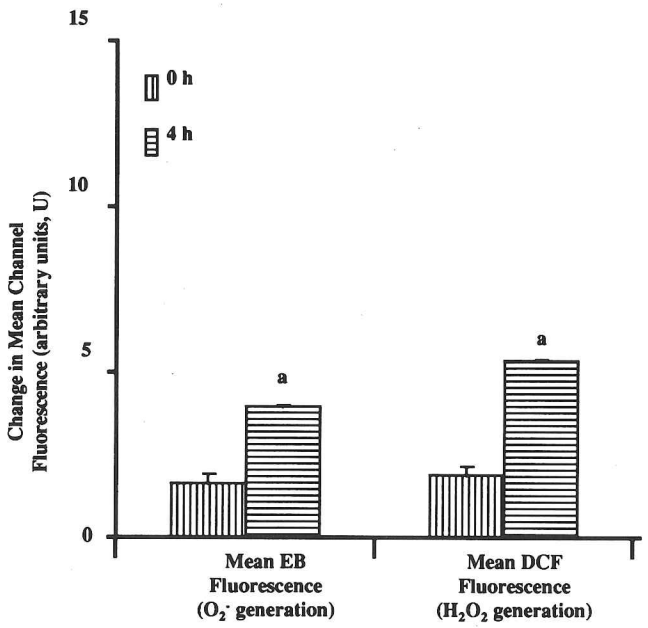

Fig. 2. O_2^- and H_2O_2 production, in neutrophils at 0 and 4 h. A significant increase in O_2^- and H_2O_2 production was seen in neutrophils at 4 h when compared with time zero in the absence of any external stimulus. [n = 3; a, significantly different from neutrophils at time zero ($P < 0.05$)].

39.35 ± 1.20 U (**Fig. 4A**). Even though there was no significant difference in O_2^- generation in neutrophils in the HLS region at 12 h when compared with neutrophils at the zero time point, neutrophils in the LLS region demonstrated a significantly reduced ($P < 0.001$) capability for O_2^- generation. At 48 h, neutrophils in both HLS (1.12 ± 0.07 U) and LLS regions exhibited a significant reduction in O_2^- generation when compared with cells at 0, 12, and 24 h.

PMA-induced intracellular H_2O_2 production in neutrophils was detected by the increase in mean channel DCF fluorescence at 30 min [(mean ± SEM fluorescence units (U)]. In the case of PMA-stimulated neutrophils, a significant reduction ($P < 0.001$) in intracellular PMA-induced H_2O_2 production was noticed in aging neutrophils at 12 h (HLS region; 112.9 ± 0.28 U) when compared with H_2O_2 production in neutrophils at 0 h (159.15 ± 1.63; Fig. 4B). Intracellular H_2O_2 production in the LLS region showed a significant reduction (11.55 ± 1.0 U) when compared with H_2O_2 in the HLS region at 12 h. A progressive reduction in intracellular H_2O_2 production was observed at 24 and 48 h, respectively. Mean DCF fluorescence at 24- and 48-h time points were 98.25 ± 1.34 U (HLS region), 13.7 ± 0.9 U (LLS region), 5.86 ± 0.81 U (HLS region), and 2.19 ± 0.76 U (LLS region), respectively.

To determine whether the reduction in H_2O_2 production reflected an alteration in the functional properties of SOD, the X-XO system was employed initially to generate O_2^- detected by increasing absorbance at 550 nm caused by the reduction of ferricytochrome *c* [31]. The total amount of SOD in freshly isolated neutrophils was 40 ± 2.0 ng/10^7 cells (**Table 1**). At the end of the 24-h incubation period total SOD activity declined significantly ($P < 0.001$) to 10.16 ± 2.15 ng/10^7 cells. Potassium cyanide inhibited the activity of SOD isolated in this manner in a dose-dependent manner, with more than half-maximal inhibition at 1 mM, indicating that the type of SOD under observation was indeed Cu,Zn-SOD (data not shown).

Intracellular GSH content

GSH labeling was determined by subtracting the background staining obtained after GSH depletion by NEM from total mBrB fluorescence as described by Hedley and Chow in 1994 [27]. Neutrophils showed a progressive decline in GSH content when incubated at 37°C for 12, 24, and 48 h, respectively (**Fig. 5**). There was a significant decrease in GSH content ($P < 0.05$) in the HLS region at 12 h (9.90 ± 0.04 U) when compared with neutrophils at 0 h (14.25 ± 0.21 U). As the incubation period progressed to 24 and 48 h, neutrophils demonstrated a gradual decrease in intracellular GSH content when compared with control ($P < 0.001$) at 12 h. Neutrophils in the LLS region showed a significant decrease in GSH content, 0.79 ± 0.92, 1.05 ± 0.90, and 0.41 ± 0.75 U, at 12, 24, and 48 h, respectively, when compared with cells in the HLS region at both 0 and 12 h.

DISCUSSION

Extensive diversity exists among signals and metabolic events necessary to initiate apoptosis in various cell types.

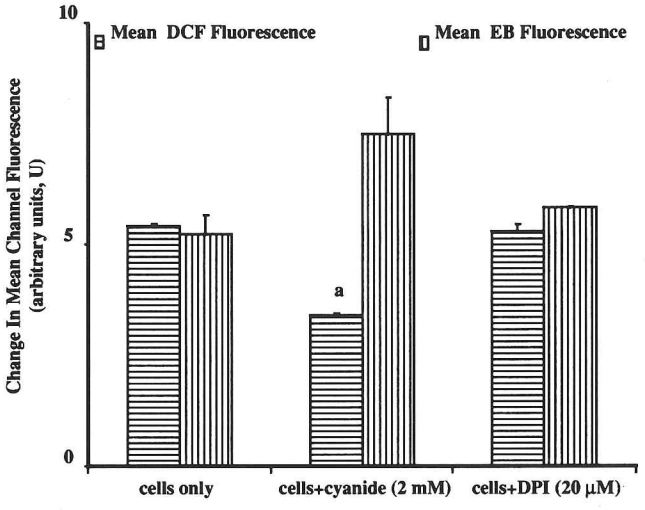

Fig. 3. O_2^- and H_2O_2 production in neutrophils at 4 h in the presence and/or absence of cyanide (2 mM) and DPI (20 μM). A significant decrease in H_2O_2 production was seen in neutrophils at 4 h when exposed to cyanide for 30 min. A nonsignificant increase in O_2^- generation is due to the inhibitory action of cyanide on cytochrome *c* oxidase and Cu,Zn-SOD (SOD inhibition prevents dismutation of O_2^- to H_2O_2). DPI, a NADPH oxidase inhibitor, did not alter ROS production. [n = 3; a, significantly different from neutrophils without cyanide at 4 h ($P < 0.05$)].

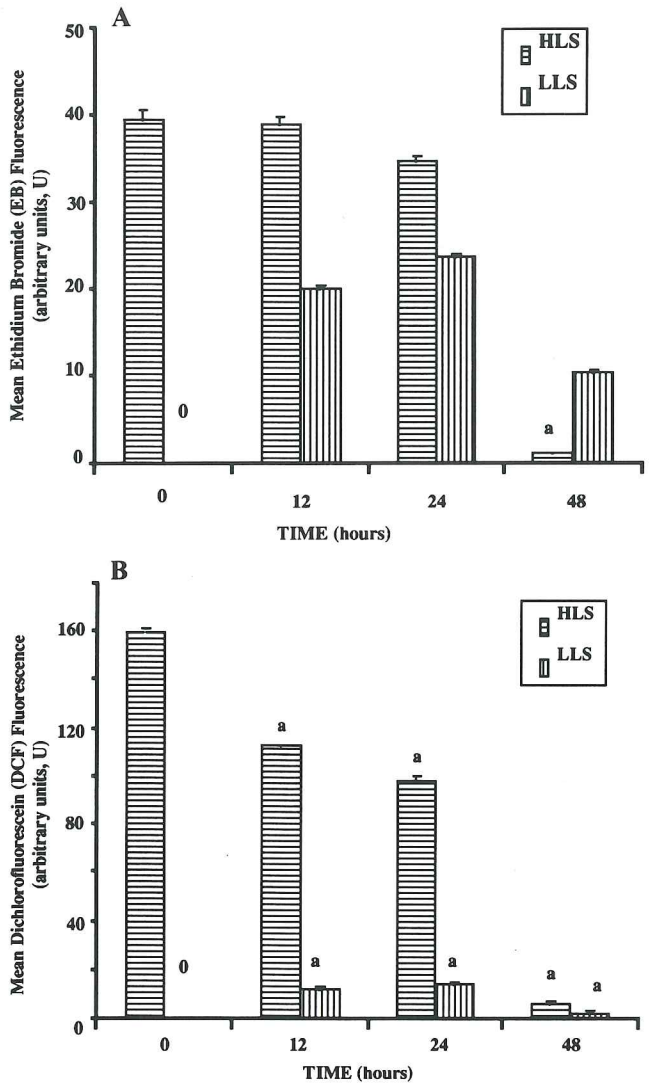

Fig. 4. (A) O_2^- production in neutrophils at 0, 12, 24, and 48 h in response to PMA stimulation. No significant changes were observed in O_2^- production in neutrophils in the HLS region at 0-, 12-, and 24-h time points when exposed to 10 ng/mL PMA. Neutrophils in the LLS region produced significantly less O_2^- when compared with neutrophils in the LLS region. There was no significant difference between PMA stimulation in neutrophils in the LLS region at 12- and 24-h time points. The significant reduction observed in O_2^- production at 48 h in both the regions was primarily due to the higher percentage of dead cells in the neutrophil population at that time. [$n = 3$; a, significantly different from neutrophils at 0, 12, and 24 h ($P < 0.05$)]. (B) H_2O_2 production in neutrophils at 0, 12, 24, and 48 h in response to PMA stimulation. Significant changes were noticed in H_2O_2 production measured in terms of DCF fluorescence in neutrophils in the HLS region at all time points when exposed to 10 ng/mL PMA. Neutrophils in the LLS region produced significantly less H_2O_2 when compared with neutrophils in the HLS region. There was no significant difference between PMA stimulation in neutrophils in the LLS region at 12- and 24-h time points. The significant reduction observed in H_2O_2 production is primarily due to the inactivation of SOD in neutrophils undergoing apoptosis. [$n = 3$; a, significantly different from neutrophils at time zero ($P < 0.05$)].

Based on the evidence presented above, we propose that neutrophil apoptosis is in part related to the ability of the cell to maintain an appropriate oxidant-antioxidant balance. Oxygen, in its role as a terminal acceptor for electrons in oxidative phosphorylation, engenders a variety of ROS, including O_2^-, H_2O_2, and OH· and its higher-energy singlet states [35]. Intracellular sources of ROS include mitochondrial oxidation, the microsomal cytochrome *P*450 system, and plasma membrane NADPH oxidases [36]. The primary ROS generating system in activated neutrophils is the plasma membrane NADPH oxidase complex, whereas the mitochondria serve as the primary source in the dormant state [37, 38].

TABLE 1. Inactivation of Cu,Zn-SOD in Aging Neutrophils

Condition	SOD content (mean ± SE)/10^7 cells
Neutrophils (0 h)	40.00 ± 2.0 ng
Neutrophils (24 h)	10.16 ± 2.15 ng ($P < 0.001$)

SOD-inhibitable ferricytochrome *c* reduction assay was employed to detect SOD activity in neutrophils at 0 and 24 h.

Our findings indicate that basal O_2^- and H_2O_2 production in neutrophils isolated from peripheral blood and cultured in vitro increases as early as 4 h. The nature of the possible mechanism responsible for this increase in O_2^- and H_2O_2 production in unstimulated neutrophils is most likely related to uncoupling of the mitochondrial electron transport system, which leads to increased free radical generation. This conclusion was supported by studies employing cyanide, an inhibitor of cytochrome *c* oxidase, and DPI, an inhibitor of the plasma membrane NADPH oxidase [39]. The insignificant increase in O_2^- production upon cyanide inhibition was due to (1) inhibition of cytochrome *c* oxidase, which resulted in residual O_2 reduction and increased O_2^- production, and (2) inhibition of cyanide-sensitive Cu,Zn-SOD, which is already undergoing a reduction in its capacity to dismutate O_2^- to H_2O_2. Electron transport throughout the mitochondrial respiratory chain accounts for the vast majority of O_2 consumption in resting neutrophils [37]. Mitochondria have previously been identified as an important source of ROS in reductive stress in rat hepatocytes in the presence of oxygen [40]. Similar findings were observed in sympathetic neurons undergoing apoptosis when deprived of nerve growth factor (NGF). Production of ROS peaked 3 h after deprivation of NGF [41].

Superoxide is a poorly reactive radical and is lipid insoluble. To dissipate more damage, O_2^- must be converted to a more reactive radical such as OH· achieved by the Fe^{2+} or Cu^{2+} catalyzed Haber-Weiss reaction. Several superoxide-dependent phenomena have been shown to result from OH· produced secondarily from superoxide via this reaction, including bactericidal action by leukocytes [35, 42]. Production of OH· results in abstraction of a hydrogen atom from the carbon chain of an unsaturated fatty acid and can initiate a chain reaction leading to peroxidation of a large number of unsaturated fatty acids. Lipid peroxidation affects the physical stability of the membranes, resulting in altered calcium homeostasis, activation of endonucleases, oxidative damage to DNA, and sulfhydryl modification of proteins [43, 44].

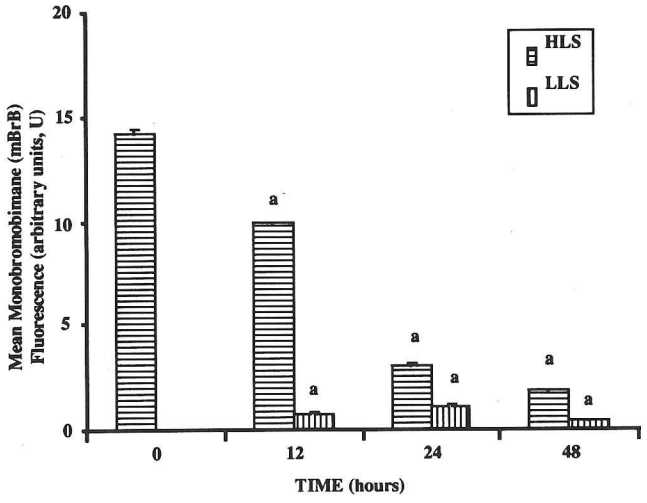

Fig. 5. Intracellular GSH content in neutrophils at 0, 12, 24, and 48 h. Intracellular GSH content measured in terms of mBrB fluorescence shows a significant decrease in neutrophils in the HLS region at all time points. Neutrophils in the LLS region had significantly less GSH, which indirectly indicates low cell viability in that region. All measurements were made in triplicates. [$n = 3$; a, significantly different from neutrophils at time zero ($P < 0.05$)].

Concomitant with the elaboration of ROS in neutrophils undergoing apoptosis, significant changes in morphology take place, reflected as changes in light scattering properties, a measurement entirely unique to flow cytometry. Light scatter changes coupled with the ability of live cells to exclude PI were exploited to assess the viability and ROS-producing capacity of neutrophils falling into individual gates assigned previously. The plasma membrane of cells undergoing apoptosis remains intact [17]. Neutrophils falling into the HLS region consistently expressed high viability (80–100%) at all time points except the 48-h time point, where the viability fell to 64%. In contrast, neutrophils in the LLS region were less viable as demonstrated by an increased membrane permeability to PI.

Human neutrophils cultured under conditions similar to those described above undergo time-related loss of cytoskeletal functions, phagocytosis, degranulation, and respiratory burst in direct relationship to onset of apoptosis [22]. Respiratory burst measured in terms of extracellular generation of O_2^- demonstrated that O_2^- production to the receptor-independent stimulus, PMA, was preserved in apoptotic neutrophils, implying some retention of intracellular signaling pathways relevant to superoxide production [22]. Our studies mainly centered around measuring the intracellular PMA-stimulated generation of O_2^- via oxidation of HE and of H_2O_2 by oxidation of DCF. Intracellular O_2^- generation in neutrophils in the HLS region did not show any significant change over the period of observation. The above findings concur with the observations of Whyte et al. [22], which showed that there was no difference in O_2^- generation between freshly isolated and aged (24 h) neutrophils poststimulation with 100 nM PMA. However, viable neutrophils in the LLS region, with a high percentage of dead cells, showed a significant reduction in O_2^- production on a per live cell basis when compared with neutrophils in the HLS region at all time points.

A significant down-regulation of PMA-stimulated H_2O_2 production was seen at 12 h in neutrophils in the HLS region when compared with H_2O_2 production in freshly isolated neutrophils at 0 h. The diminished H_2O_2 production in aging neutrophils is secondary to inactivation of intracellular SOD, leading to decreased dismutation of superoxide via SOD. We were able to demonstrate that SOD content in supernatants of neutrophil lysates decreased fourfold on aging. SOD undergoes oxidative modification and inactivation following exposure to OH· radicals [45]. Analysis of the mechanism by which stimulated neutrophils inactivate SOD revealed that the cascade of the oxidative reaction of neutrophils is responsible for SOD inactivation [46]. A role for ROS in inducing apoptosis is evident at the genetic level in neuronal disorders such as familial amyotrophic lateral sclerosis. Oxygen radical-mediated loss of motor neurons in the spinal cord and neocortex results from mutations within Cu,Zn-SOD [47]. Chronic inhibition of SOD by antisense oligonucleotides or metal chelating agents such as diethyldithiocarbamate also produces apoptotic death of spinal neurons [48, 49]. Inhibiting the synthesis of Cu,Zn-SOD by antisense oligonucleotides in untreated and NGF-treated PC12 cells decreased cell survival, with NGF-treated cells dying at lower levels of inhibition than untreated cells [49]. Cultured sympathetic neurons injected with Cu,Zn-SOD protein or with an expression vector containing SOD cDNA also showed a delay to undergo apoptosis [41].

GSH is a major intracellular reducing agent involved in numerous metabolic processes [27]. The primary role of GSH as an antioxidant is to reduce the intracellular H_2O_2 load. It is capable of preserving cell integrity by reducing intracellular peroxides and free radicals, as well as by maintaining protein disulfide bonds in the reduced state [50]. The scarcity of GSH in HIV infection and AIDS promotes apoptosis mediated by oxidant stress [36, 51]. Prolonged incubation at 37°C produces ROS, which warrants the concerted effort of the antioxidant defense mechanisms in the cell. A progressive decrease in GSH content can be seen as early as 4 h (data not shown), which indicates that cells may be already under early oxidative stress.

In summary our data indicate that ROS molecules generated in vitro may participate as effector molecules during the early events of apoptosis. The apparent source of these ROS is the mitochondrial respiratory chain. Stress-induced uncoupling of mitochondrial respiration in vitro leads to a concomitant decrease in mitochondrial transmembrane potential and ATP synthesis [52–54]. Zamzami et al. [52] reported that alteration of mitochondrial function is an early feature of apoptosis. A sequential disregulation of mitochondrial function associated with a reduction of mitochondrial transmembrane potential and increased ROS production (detected by conversion of HE to EB) was found in U937 and WEHI-231 pre-B cells. These in vitro models had nontoxic agents such as tumor necrosis factor α, anti-immunoglobulin M, CD3 cross-linking, and ceramide-

inducing apoptosis in U937 cells, WEHI 231 pre-B cells, and T cell hybridomas, respectively. In all these systems, alterations in mitochondrial function preceded DNA fragmentation and nuclear DNA loss [52].

Onset of apoptosis in neutrophils is much more complex than the simple mechanism we have studied here, but the role of oxidative stress proposed here can also account for the spontaneous induction of neutrophil apoptosis in vitro. An increase in ROS generation has self-perpetuating properties, in the sense that decreased SOD activity and GSH content resulting from incessant stress can spread in a wave-like fashion, leading to elevated cytosolic calcium levels that can directly or indirectly aid in cell death via apoptosis [36].

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