THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 29, Issue of July 21, pp. 22461–22469, 2000

Printed in U.S.A.

Myeloperoxidase Is Involved in H_2O_2-induced Apoptosis of HL-60 Human Leukemia Cells*

Received for publication, February 22, 2000, and in revised form, April 28, 2000
Published, JBC Papers in Press, May 3, 2000, DOI 10.1074/jbc.M001434200

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We examined the mechanism of H_2O_2-induced cytotoxicity and its relationship to oxidation in human leukemia cells. The HL-60 promyelocytic leukemia cell line was sensitive to H_2O_2, and at concentrations up to about 20–25 μM, the killing was mediated by apoptosis. There was limited evidence of lipid peroxidation, suggesting that the effects of H_2O_2 do not involve hydroxyl radical. When HL-60 cells were exposed to H_2O_2 in the presence of the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), we detected a 12-line electron paramagnetic resonance spectrum assigned to the POBN/POBN$^{\cdot}$ N-centered spin adduct previously described in peroxidase-containing cell-free systems. Generation of this radical by HL-60 cells had the same H_2O_2 concentration dependence as initiation of apoptosis. In contrast, studies with the K562 human erythroleukemia cell line, which is often used for comparison with the HL-60, and with high passaged HL-60 cells (spent HL-60) studied under the same conditions failed to generate POBN$^{\cdot}$. Cellular levels of antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase did not explain the differences between these cell lines. Interestingly, the K562 and spent HL-60 cells, which did not generate the radical, also failed to undergo H_2O_2-induced apoptosis. Based on this we reasoned that the difference in H_2O_2-induced apoptosis might be due to the enzyme myeloperoxidase. Only the apoptosis-manifesting HL-60 cells contained appreciable immunoreactive protein or enzymatic activity of this cellular enzyme. When HL-60 cells were incubated with methimazole or 4-aminobenzoic acid hydrazide, which are inhibitors of myeloperoxidase, they no longer underwent H_2O_2-induced apoptosis. Hypochlorous acid stimulated apoptosis in both HL-60 and spent HL-60 cells, indicating that another oxidant generated by myeloperoxidase induces apoptosis and that it may be the direct mediator of H_2O_2-induced apoptosis. Taken together these observations indicate that H_2O_2-induced apoptosis in the HL-60 human leukemia cell is mediated by myeloperoxidase and is linked to a non-Fenton oxidative event marked by POBN$^{\cdot}$.

Neoplastic and normal cells produce H_2O_2 in the mitochondria, cytosol, and peroxisomes during physiologic processes as a product of intracellular oxidases and superoxide dismutase (SOD)[1] (1). H_2O_2 is an important intracellular compound that influences cellular redox state (2), acts as or generates signaling molecules (1, 2), and modulates gene expression (3). However, as a reactive oxygen species, H_2O_2 can cause tissue injury in many cell types by both apoptosis and necrosis (4). In this regard, H_2O_2 has been implicated as a possible intracellular mediator in the toxicity of external stimuli such as ultraviolet radiation (5–8), ionizing radiation (9–11), hematoporphyrin-mediated photodynamic therapy (12) and chemotherapy (13–15). This cytotoxicity is likely mediated by some oxidative event. H_2O_2 traverses membranes almost as rapidly as water. Because it is only a weak oxidizing agent (kinetically), it is generally assumed that it reacts with Fe^{2+} or Cu^{+} to form $^{\cdot}OH$ via the Fenton reaction (16, 17).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \qquad \text{(Eq. 1)}$$

It is often assumed that this one-electron reduction is the pathway to explain H_2O_2 toxicity (18). However, we do not know if H_2O_2 itself or an oxidative product such as $^{\cdot}OH$ targets membranes to react with polyunsaturated fatty acids or cholesterol (19) or causes oxidation of DNA or proteins to trigger cytotoxicity.

In phagocytes, microbicidal activity depends upon toxic oxygen-derived products such as H_2O_2, HOCl derived from H_2O_2, and subsequent oxidizing species such as chloramines and $^{\cdot}OH$ (20). HOCl is generated by H_2O_2 in the presence of chloride and the granule protein myeloperoxidase (MPO) (donor: H_2O_2 oxidoreductase, EC 1.11.1.7).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O \qquad \text{(Eq. 2)}$$

Oxidative modification by this H_2O_2/MPO/halide system may play a part in inflammatory, neurologic, neoplastic, and vascular disease. One specific marker of myeloperoxidase-related oxidation, 3-chlorotyrosine, has been isolated from low density lipoprotein in human atherosclerotic lesions at considerably higher concentrations than in normal aorta (21). Furthermore, chlorotyrosine levels were higher *in vivo* in the bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome than those in control patients, and the amounts correlated with myeloperoxidase concentration (22).

Apoptosis, or programmed cell death, may be initiated by diverse stimuli, some of which are pathologic and some of

* This investigation was supported by Grant P01 CA66081 awarded by the National Cancer Institute, Department of Health and Human Services; The Iowa Leukemia and Cancer Research Fund; The Dr. Richard O. Emmons Memorial Fund; and The Mamie C. Hopkins Fund. 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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[1] The abbreviations used are: SOD, superoxide dismutase; POBN, [α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone]; MPO, myeloperoxidase; MPO-I, MPO-II, MPO compounds I and II; FBS, fetal bovine serum; EPR, electron paramagnetic resonance spectroscopy; TBARS, thiobarbituric acid reactive substances.

which are part of the normal processes of development and homeostasis in living organisms. We have a partial understanding of the signaling events that precede the morphologic changes characteristic of apoptosis; however, we do not understand how chemical stimuli such as H_2O_2 result in the induction of apoptosis (23–25). For many agents that induce apoptosis, the target(s) or effectors that transduce the initiation event are unidentified. There is evidence that reactive oxygen species such as H_2O_2 may play a role in this process especially with selected initiators, because antioxidants inhibit apoptosis (26–28). In fact, both forms of cell death, apoptosis and necrosis, can be blocked by antioxidants (29).

In the present study we investigated the oxidative events that are associated with H_2O_2-induced apoptosis in the human HL-60 leukemia cell. We found that the generation of a POBN free radical correlated with programmed cell death in human leukemia cells and both seemed to be mediated by cellular MPO.

EXPERIMENTAL PROCEDURES

Cell Culture and Membrane Damage Assessment—HL-60 and K562 cells (obtained from the American Type Tissue Culture, Rockville, MD) were cultured at 37 °C in humidified air in RPMI 1640 with 10% fetal bovine serum (FBS; Life Technologies, Inc.) and supplemented with 2 mM L-glutamine, 85 units/ml penicillin, and 85 μg/ml streptomycin. HL-60 is a human myeloid leukemia cell that grows as a suspension culture (30). HL-60 cells produce MPO that has the same electrophoretic behavior as MPO found in normal human neutrophils (31). HL-60 cells can be induced by chemicals and drugs to differentiate, but in this study the undifferentiated line was used. It is known to undergo apoptosis when exposed to anticancer agents (32). High-passaged HL-60 cells that had lost much of their biological versatility, including the ability to undergo H_2O_2-induced apoptosis, are referred to as spent HL-60 cells. These cells were passed greater than 85 times, and at about that point they began to change biologically. K562 cells are a human myeloid leukemia cell line derived from the bone marrow of a patient with chronic myelogenous leukemia (33). K562 cells grow as a suspension culture and are often used for comparison to HL-60 (34). Cells in the log phase (48-h cultures) were washed and placed in the above media with the cell density adjusted to 10×10^6/ml (electron paramagnetic resonance spectroscopy (EPR) studies) or 0.5×10^6/ml (apoptosis studies). All experiments were done in the presence of RPMI 1640 plus 10% fetal calf serum unless specified otherwise. Cells were counted with a cell counter (model Zf; Coulter, Inc., Hialeah, FL). Trypan blue dye exclusion was used to assess membrane damage during exposures to H_2O_2.

DNA Fragmentation Analysis by Agarose Gel Electrophoresis—After treatment, cells were washed and resuspended in phosphate-buffered saline, and a cell pellet was formed by centrifugation at $500 \times g$ in an Eppendorf Model 5415C centrifuge. The supernatants were removed, and 330 μl of Sarkosyl detergent lysis buffer (50 mM Tris, 10 mM EDTA, 0.5% w/v sodium-*N*-lauroyl sarcosine, pH 7.5) and 13 μl of Proteinase K (15.6 mg/ml) (Roche Molecular Biochemicals GmbH, Germany) were added, vortexed, and allowed to digest overnight at 37 °C. RNase A (0.3 mg/ml) was added, and the samples were incubated for 1 h at 56 °C. The lysates were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), vortexed, and centrifuged at $16,000 \times g$ for 5 min. The upper phase containing the DNA was transferred to new tubes, 40 μl of 3 M sodium acetate and 1 ml of 100% ethanol were added, and the samples were kept overnight at −20 °C to precipitate DNA. The sample was then centrifuged at $16,000 \times g$ for 10 min, the supernatant was removed, and 0.5 ml of 70% ethanol was added to the DNA pellet. The sample was centrifuged for 5 min at $16,000 \times g$, and the supernatant was removed. The DNA pellet was then placed under a vacuum until a dry pellet was obtained. The DNA was solubilized in Tris/EDTA (10 mM/1 mM) buffer and mixed. The resultant DNA in solution was then quantitated spectrophotometrically at 260/280 nm. Total DNA (2 μg) was mixed with 10× BlueJuice gel loading buffer (Life Technologies, Inc.) and tracking dye and loaded on 1% agarose gels containing ethidium bromide. Gels were electrophoresed for 2.5 h at 50 V, destained with water, and illuminated with ultraviolet light for examination and photography.

Morphological Assessment of Apoptosis by Cytospin Preparations—Following experimental exposures to oxidants, cells underwent a 24-h additional incubation at 37 °C, and then aliquots were centrifuged for 5 min at $300 \times g$. The cell pellets were then resuspended in 0.9% NaCl with 0.5 mg/ml bovine serum albumin. Cytospin preparations were made with the resuspended cells. Dried slide preparations were then stained with Wright's stain, and morphological evaluations were made by light microscopy. Triplicate 100-cell counts on consecutive cells in at least three regions of each slide from different samples were done to determine the percentage of apoptotic cells. The morphologic features of apoptosis in the HL-60 cells have been described (35). Cells were scored as apoptotic if there was clear expression of either densely condensed nuclear chromatin, including chromatin collapsed down into crescents along the nuclear envelope, or apoptotic bodies. If these features were absent, then both cell shrinkage and membrane blebbing were required.

MPO Assay—Peroxidase activity was determined by the ability to oxidize tetramethylbenzidine (36, 37). Briefly, cells were washed twice using 0.9% NaCl, and the cell density was adjusted to 1×10^6 cells/ml using the Coulter Model Zf cell counter. Cells (200,000 total) were then placed in 3.05 ml of MPO assay buffer (50 mM sodium acetate, pH 5.4) and sonicated for 10 s with an ultrasonic probe. The cellular lysate was equilibrated to room temperature, then 50 μl of 100 mM 3,3′,5,5′-tetramethylbenzidine in dimethylformamide was added and the assay was initiated with 200 μl of 5.25 mM H_2O_2 in assay buffer. The sample was mixed and allowed to incubate for 3 min prior to the addition of 100 μl of catalase (0.3 mg/ml in water), then 3.4 ml of ice-cold 200 mM acetic acid in water was added to quench the reaction. The samples were briefly centrifuged to remove cell debris, and the absorbance was read at 655 nm.

Western Blot Analysis—Washed cells were sonicated on ice (three times for 30 s each) using a Vibra Cell cup horn sonicator (Sonics and Materials, Inc., Newtown, CT) at maximum power. Protein from 1.5×10^5 cells was placed in each well, separated by electrophoresis on 12.5% polyacrylamide gels (38), and subjected to Western analysis. Briefly, separated proteins were transferred onto nitrocellulose membranes and blocked in 5% dry milk in 0.01 M Tris/0.15 M NaCl buffer, pH 8.0, and 0.1% Tween 20. Blots were rinsed three times and incubated with MPO antibodies for 1 h at room temperature. Monospecific rabbit polyclonal antiserum against MPO was acquired from Dr. William M. Nauseef (39) and diluted 1:500 for use. After further washing, the blot was incubated in goat anti-rabbit IgG conjugated with horseradish peroxidase at a 1:10,000 dilution for 1 h at room temperature. Blots were washed again, and bands were visualized using chemiluminescence.

Detection of Radicals—To a HL-60 cell suspension (10×10^6/ml) in RPMI 1640/10% FBS were added 50 mM POBN and then H_2O_2. Immediately, an aliquot of the sample was placed into an EPR quartz flat cell and positioned in a TM_{110} cavity of a Bruker ESP-300 EPR spectrometer. The EPR scans were initiated in the "additive" mode for 80 consecutive scans, with approximately 55 min of monitoring time. To monitor $^{\cdot}OH$ radical production, as hydroxyethyl radical adducts, experiments were set up as described above but with the addition of 1% ethanol. EPR instrument settings were as follows: 40-milliwatt microwave power at a frequency of 9.78 GHz; modulation frequency of 100 kHz; receiver gain 2.5×10^5; modulation amplitude 0.7 G; and scanning rate 50 G/42 s with a time constant of 20.5 ms. Spin adduct concentration estimates were made using 3-carboxyproxyl (Aldrich) as a standard (40).

Thiobarbituric Acid Reactive Substances—Cell samples were pelleted at $300 \times g$ for 10 min and washed once with phosphate-buffered saline and additionally with 0.9% NaCl. The sample pellet was suspended in 2 ml of 0.9% NaCl, and then butylated hydroxytoluene in ethanol (385 μM final concentration) was added to the samples, which were then frozen at −20 °C until assay. Samples were thawed and vortexed vigorously, and an aliquot was taken for a Lowry protein assay (41). To the remaining sample was added 230 μl of trichloroacetic acid-saturated solution (250 g of trichloroacetic acid to 100 ml of water), which was then vortexed, and centrifuged at $3000 \times g$ (10 min) to precipitate protein. Supernatant (1.6 ml) was placed in glass test tubes, and 200 μl of 14.4 mg/ml 2-thiobarbituric acid in 0.1 N NaOH solution was added. The samples were incubated for 30 min at 75 °C. Standards were prepared from the hydrolysis of 1,1,2,2-ethoxypropane in a 40% trichloroacetic acid solution. After cooling, the absorbances of the samples were read at 535 nm and thiobarbituric acid reactive substances (TBARS) values were calculated as both nanomoles/2.5×10^6 cells and nanomoles/mg of cell protein.

Antioxidant Enzymes—Mn-SOD and CuZn-SOD (42), catalase (43), and glutathione peroxidase (44) enzymatic activities were determined in washed cells after sonication.

Statistical Analysis—The results are expressed as mean ± S.E. Significant differences were evaluated with the unpaired Student's *t* test or one-way analysis of variance. Dunnett's test was used for pairwise

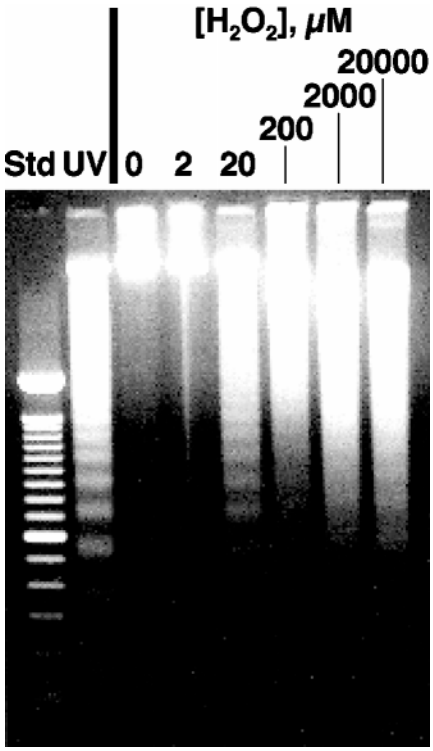

FIG. 1. **H_2O_2 induces apoptosis in HL-60 cells by DNA fragmentation analysis.** HL-60 cells were treated with H_2O_2 for 24 h, and genomic DNA was isolated, separated on a 1% agarose gel for 2.5 h at 50 V (2 μg per lane), and stained with ethidium bromide. *Lanes* from *left* to *right* are 100-base pair DNA ladder standards (Life Technologies, Inc.), HL-60 cells ultraviolet light-irradiated at 302 nm for 5 min and incubated an additional 3 h; the remaining *lanes* are HL-60 cells treated with increasing concentrations of H_2O_2 for 24 h.

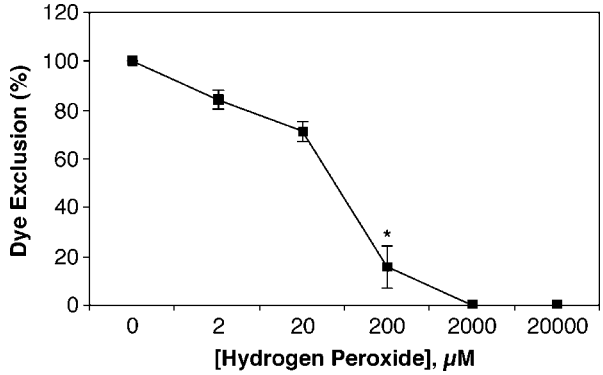

FIG. 2. **H_2O_2 increases membrane permeability.** HL-60 cells were incubated with increasing concentrations of H_2O_2 for 24 h, and membrane integrity was estimated by trypan blue exclusion. Each point represents the mean and S.E. of independent determinations from seven different experiments. The overall test of differences among the H_2O_2 concentrations was statistically significant ($p = 0.007$ by one-way analysis of variance); only the 200 μM level differed significantly from the control mean and is so indicated by the *asterisk*. The replicates at 2000 and 20,000 μM were all zero and are also likely to be significantly lower than the controls.

comparisons *versus* control (45). All statistical tests were carried out at the 5% level of significance.

RESULTS

Analysis of Apoptosis by DNA Fragmentation—H_2O_2 is a natural product of metabolism, but at sufficient concentrations it produces cell damage. To demonstrate whether H_2O_2 induces apoptosis in HL-60 cells, we treated HL-60 cells with incremental concentrations of H_2O_2 for 24 h, isolated genomic DNA, and separated it on agarose gels looking for the important hallmark of apoptosis, endonuclease fragmentation of DNA. Fig. 1 is a representative gel showing DNA laddering induced by H_2O_2. Cells incubated with 0 or 2 μM of H_2O_2 showed no evidence of DNA fragmentation. However, cells treated with 20 μM H_2O_2 showed considerable DNA fragmentation with the internucleosomal base pair fragments that were similar to those produced by UV light in the *lane 2* positive control. Studies at intermediate concentrations indicated that the threshold for apoptosis was 10–15 μm (data not shown). At >20 μM H_2O_2 concentrations, nonspecific DNA degradation predominated (loss of high molecular weight DNA and non-endonuclease-associated fragmentation of DNA resulting in smearing). This loss of high molecular weight DNA and background smearing is consistent with cell necrosis. Therefore, H_2O_2 induces apoptosis in HL-60 cells at lower concentrations beginning at 10–15 μM and necrosis at concentrations >20 μM.

Membrane Damage by H_2O_2—Increasing concentrations of H_2O_2 were added to the growth media of HL-60 cells, and after 24 h, trypan blue exclusion was measured as an estimate of membrane damage. As can be seen in Fig. 2, 85% of the cells were capable of excluding the dye at 2 μM H_2O_2 and more than 71% at 20 μM. Beginning at 200 μM H_2O_2 the value differed significantly from the control mean; therefore, at higher concentrations, the membrane became permeable. This indicates that the necrosis observed at H_2O_2 concentrations of 200 μM and above is associated with, and possibly due to, acute membrane damage.

Minimum Time of H_2O_2 Exposure Required for Apoptosis—To determine the minimum duration of H_2O_2 exposure time required to induce apoptosis in HL-60 cells, we exposed them to 20 μM H_2O_2, and then, at 1, 1.5, 2, 2.5, and 5 min, catalase (1000 units/ml) was added to remove the H_2O_2. The cells were then allowed to incubate for 24 h before the DNA was extracted for agarose gel electrophoresis. Fig. 3 shows that, at the minimum, 1.5 min of exposure to 20 μM H_2O_2 is required to induce apoptosis.

Thiobarbituric Acid Reactive Substances—To determine if lipid peroxidation has a role in H_2O_2-induced apoptosis, we evaluated the levels of TBARS from cells treated with different concentrations of H_2O_2 for 24 h (Fig. 4). There was a low level of TBARS production from H_2O_2 at all concentrations. For comparison, the greater TBARS generation from Fe^{2+} is also shown. This suggests that lipid peroxidation is not being induced to any great extent by the H_2O_2. The TBARS production was also determined as nanomoles/mg of protein, and the conclusions were similar.

It is known that TBARS are unstable in the presence of H_2O_2 (46), and such degradation could explain our low TBARS values. However, this instability is usually seen at mM concentrations and, in fact, 20 mM H_2O_2, the highest concentration we used in any experiment, degraded the malondialdehyde-thiobarbituric acid complex by less than 6% (46).

Limited Hydroxyl Radical Production by Cells—We next measured the generation of $^{\cdot}OH$ measured as hydroxyethyl adducts of POBN detected by EPR after exposing HL-60 cells to 20 μM H_2O_2 in RPMI 1640/10% FBS in the presence of 50 mM POBN under the same conditions as those for apoptosis. There was generation of hydroxyethyl adduct, but the magnitude of the adduct production was no greater than that produced by the cell-free media exposed to H_2O_2 (data not shown). This observation does not preclude the possibility of production of $^{\cdot}OH$ by the cells but only that the amount produced by the cells is not distinguishable from that produced in a cell-free environment. This can be compared with the generation of about 20-fold more $^{\cdot}OH$ when HL-60 cells are exposed to Fe^{2+}.

Generation of POBN/$^{\cdot}$POBN Adduct by HL-60 Cells Exposed to H_2O_2—We next examined other free radical events resulting

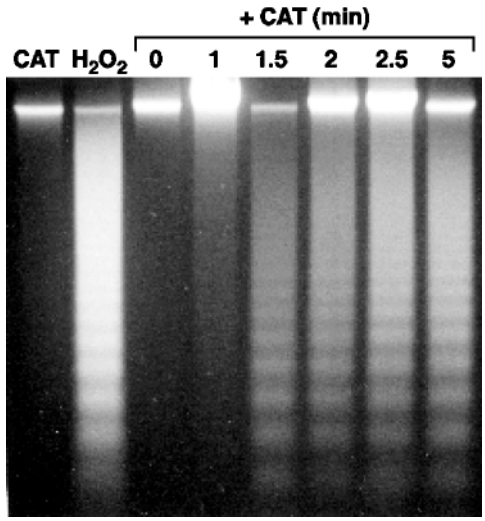

FIG. 3. **Initiation of apoptosis requires a 90-s exposure to H_2O_2.** HL-60 cells were exposed to 20 μM H_2O_2, catalase (1000 units/ml) was added at the times shown to remove H_2O_2, and then the cells were incubated at 37 °C for 24 h. Cells were harvested, DNA isolated, and laddering was examined. *Lanes* from *left* to *right* are catalase alone, H_2O_2 alone, then H_2O_2 plus catalase added at the times shown. At time 0, catalase and H_2O_2 were added simultaneously.

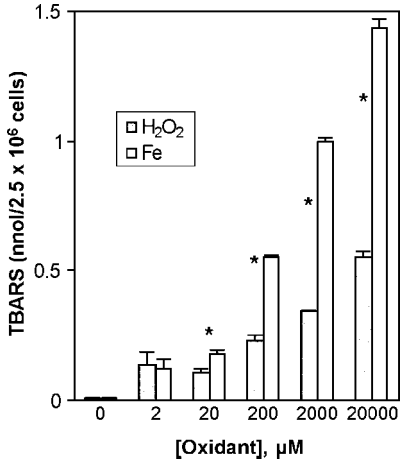

FIG. 4. **Limited production of TBARS by H_2O_2.** HL-60 cells (2.5×10^6 cells/ml) were incubated for 24 h with increasing concentrations of either H_2O_2 or, for comparison, Fe^{2+} in RPMI 1640 plus 10% FBS, then TBARS were determined. Values expressed as nmol per 2.5×10^6 cells are the means ± S.E. of samples from three separate determinations and have been normalized to 0 μM to represent the specific production resulting from H_2O_2 or Fe^{2+}. The TBARS production by H_2O_2 was significantly lower than Fe^{2+} at all concentrations above 2 μM (20 μM, $p = 0.03$; 200 μM, $p = 0.0001$; 2000 and 20,000 μM, $p < 0.0001$) as indicated by *asterisks*.

from H_2O_2 exposure using the same conditions as those for apoptosis. Fig. 5 shows representative EPR spectra obtained from HL-60 cells treated with different concentrations of H_2O_2 in the presence of the spin trap 4-POBN. Unexpectedly, a 12-line spin adduct was observed. It had a concentration threshold of 20 μM H_2O_2 and was not detected at 2 μM or in the absence of H_2O_2. There was no concentration dependence above 20 μM. This signal has a configuration ($a^N_1 = 14.95$ G, $a^N_2 = 1.56$ G, $a^H = 1.85$ G) that is compatible with the POBN/POBN$^\cdot$ adduct previously described in a cell-free system (37). In addition, human MPO, H_2O_2, and POBN in the absence of cells generate a strong EPR signal identical to the one observed in the cells, and this can be blocked by methimazole (spectra not shown). In our studies with HL-60 cells, the radical depends upon the presence of POBN and H_2O_2, but it was eliminated if

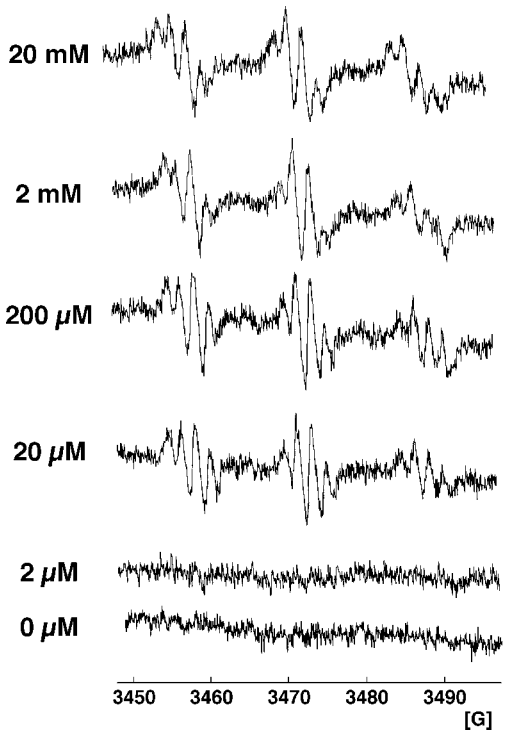

FIG. 5. **Spin trapping: POBN/POBN$^\cdot$ radical adduct is generated by exposure of HL-60 cells to H_2O_2.** Results are representative spectra from one of at least three independent experiments. HL-60 cells (10×10^6) were exposed to increasing concentrations of H_2O_2 in the presence of 50 mM POBN in RPMI 1640/10% FBS.

catalase (1000 units/ml) was present. This signal was not observed in the absence of cells. Using 3-carboproxyl standards, we estimate that the maximal concentration of the POBN/POBN$^\cdot$ adduct is 60–80 nM. HL-60 cells studied in RPMI 1640 alone or normal saline without serum also generated the POBN/POBN$^\cdot$ when exposed to H_2O_2. It is not known whether the POBN$^\cdot$ is a determinant of H_2O_2 cytotoxicity or a marker of a resultant oxidative event. In any case, the overall pattern of radical generation from increasing concentrations of H_2O_2 quantitatively paralleled that of apoptosis (Figs. 1 and 5), suggesting a relationship exists.

Lack of POBN$^\cdot$ Generation by K562 Cells and Spent HL-60 Cells—We next studied human erythroblastic K562 cells, a line often used for comparison to HL-60 cells. When exposed to 20 μM H_2O_2 under identical conditions as the HL-60 cell, there was no generation of POBN/POBN$^\cdot$ adduct by the K562 cells; however, a small carbon-centered radical consistent with a lipid-derived radical was detected (47, 48) (Fig. 6). A loss of the ability to generate POBN$^\cdot$ was also noted in a high passaged HL-60 cell line referred to as spent HL-60 cells. This latter observation provides a particularly appropriate comparison, because the spent HL-60 line has the same lineage as the HL-60 line.

Antioxidant Enzyme Activity—Table I shows the activity of the major antioxidant enzymes in the three cell types. There were few statistically significant differences. The content of Mn-SOD was higher in the HL-60 cells than that of the K562 ($p = 0.005$) cells but not of the spent HL-60 cells. Likewise, the cellular content of catalase was significantly higher in the HL-60 cells compared with K562 ($p = 0.0001$) and spent HL-60 cells ($p = 0.0002$). However, these differences were small, and except for the small differences in catalase, they were not corroborated by statistical analysis when expressed as activity per cell number rather than per milligram of protein. Thus, it seems unlikely that antioxidant enzymes explain the differences in radical generation or the apoptosis observations.

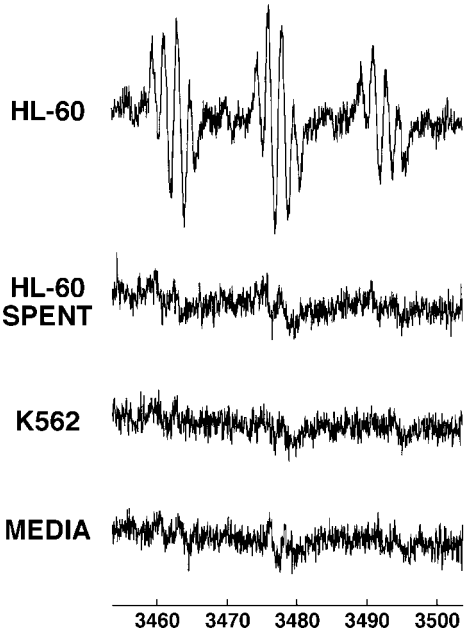

FIG. 6. **Lack of POBN/POBN$^{\cdot}$ radical adduct from K562 and spent HL-60 cells.** Shown is the spin trapping of a small carbon-centered lipid-derived radical when K562 cells, spent HL-60, and cell-free media were exposed to 20 μM H_2O_2 in RPMI 1640/10% FBS in the presence of 50 mM POBN.

TABLE I
Antioxidant enzyme activity of the cells

Enzyme	Activity		
	HL-60	K562	Spent HL-60
SOD,[a] total, units/mg	78.4 ± 10.8	40.7 ± 9.6	63.4 ± 16.9
(units/10^6 cells)	(9.9 ± 1.4)	(8.8 ± 2.1)	(7.7 ± 2.1)
CuZn-SOD, units/mg	42.6 ± 13.0	22.2 ± 8.7	36.2 ± 14.7
(units/10^6 cells)	(5.4 ± 1.6)	(4.8 ± 1.9)	(4.4 ± 1.8)
Mn-SOD, units/mg	35.1 ± 2.3	18.5 ± 1.9	27.2 ± 2.2
(units/10^6 cells)	(4.4 ± 0.3)	(4.4 ± 0.4)	(3.3 ± 0.3)
Glutathione peroxidase, units/g	4.4 ± 1.3	2.6 ± 0.3	7.4 ± 1.2
(μunits/10^6 cells)	(614 ± 243)	(440 ± 110)	(950 ± 237)
Catalase, *k*/g	25383 ± 482	10261 ± 276	18901 ± 173
(*k*/10^6 cells)	(3.1 ± 0.06)	(2.71 ± 0.07)	(2.31 ± 0.02)

HL-60, spent HL-60, and K562 cells were washed and homogenized in assay buffer. Enzymes were assayed by the methods referenced under "Experimental Procedures." Values are mean and S.E. of three to five replicates and are expressed per amount of protein. *k* is catalytic rate constant. Based on units/mg, the values for total and CuZn-SOD were not significantly different in any of the three lines. The values for Mn-SOD were significantly higher in the HL-60 cells compared to K562 ($p = 0.005$), but not the spent HL-60 cells ($p = 0.07$). The values for glutathione peroxidase were not significantly different in the three lines. The values for catalase were significantly higher in the HL-60 cells compared to K562 ($p = 0.0001$) and spent HL-60 cells ($p = 0.0002$).

[a] In this assay, 1 unit of SOD activity corresponds to 8 ng of pure bovine CuZn-SOD/ml of solution (42).

Lack of Apoptosis in K562 Cells and Spent HL-60 Cells—To further explore a possible relationship of the POBN$^{\cdot}$ to apoptosis, we exposed K562 and spent HL-60 cells, neither of which generate the radical, to 20 μM H_2O_2 under the same conditions that induced apoptosis in the HL-60 cells. No apoptosis by these cell lines was detected (Fig. 7). In other experiments not shown, we found no apoptosis at higher H_2O_2 concentrations, which were tested up to 2 mM. Studies with the K562 and spent HL-60 cells showed only necrosis beginning above 200 μM.

Analysis of Apoptosis by Morphology—We also analyzed apoptosis of the leukemic cells using Wright's stained smears of the cell suspensions. This complementary assessment of apoptosis confirmed, in general, the conclusions of the DNA fragmentation studies. Fig. 8 shows the percentage of apoptotic cells after 24 h of exposure of the three cell lines to increasing concentrations of H_2O_2. There was a concentration-dependent increase in apoptosis in the HL-60 cell line up to 200 μM. Neither the K562 nor the spent HL-60 cells showed appreciable apoptosis even at the higher concentrations of H_2O_2.

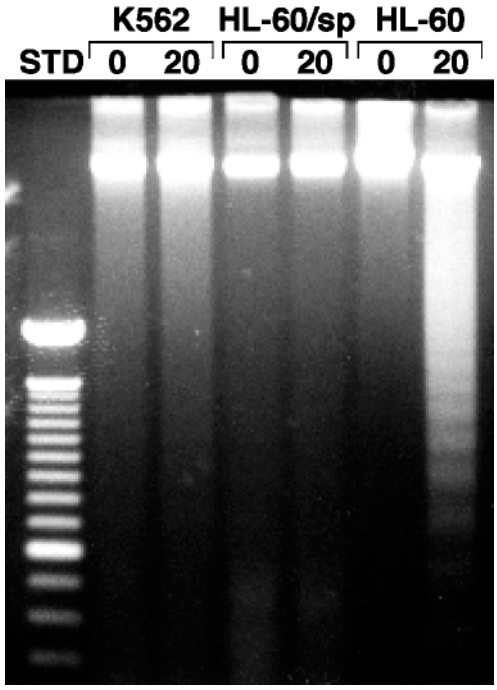

FIG. 7. **Lack of apoptosis by K562 and spent HL-60 cells.** Cells were treated with 20 μM H_2O_2 for 24 h, and apoptosis was measured by DNA fragmentation. *Lanes* are from *left* to *right* DNA ladder standard, K562 exposed to 0 μM H_2O_2, K562 exposed to 20 μM H_2O_2, spent HL-60 cells exposed to 0 μM H_2O_2, spent HL-60 cells exposed to 20 μM H_2O_2, HL-60 cells exposed to 0 μM H_2O_2, and HL-60 cells exposed to 20 μM H_2O_2.

Lack of Effect of HL-60-conditioned Media and Exogenous MPO—To test whether a soluble mediator of apoptosis was secreted by the HL-60 cells, we prepared HL-60 cell-conditioned media by incubating HL-60 cells in RPMI 1640 with 10% fetal bovine serum for 48 h prior to removal of the HL-60 cells by centrifugation. When this conditioned media was used as the growth media, K562 cells did not undergo H_2O_2-induced apoptosis as measured by DNA fragmentation. These experiments suggest that there is no soluble mediator secreted from the HL-60 cells and contained in the conditioned media that is capable of reaching a critical cellular site to mediate apoptosis. We also carried out experiments assessing the effect of exogenously added human leukocyte MPO (Sigma) on the ability of H_2O_2 to induce apoptosis by the spent HL-60 cells. Human MPO added to the medium would not support apoptosis induced by H_2O_2. Both time and concentration experiments were done. In the concentration experiments, 0, 2, 18, and 36 nM MPO was added to the medium that also contained 20 μM H_2O_2 and spent HL-60 cells. After 24-h exposure, the cells were washed and apoptosis was assessed by DNA banding. In the time experiments, a higher concentration of H_2O_2 (500 μm) was used in the presence of 14 nM MPO and spent HL-60 cells. In these experiments, it was necessary to shorten the time of exposure to avoid H_2O_2-induced necrosis, so catalase (1000 units/ml) was added at 0, 2.5, 5, and 10 min. None of these experiments resulted in apoptosis by the spent HL-60 cells. There appeared to be sufficient MPO present in the medium even 24 h after the experiment, because an aliquot of the incubation medium used for the 36 nM study had detectable MPO

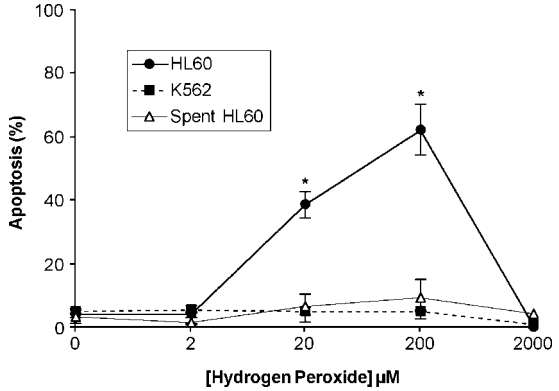

FIG. 8. **Differences in apoptosis induced by H_2O_2 in the three cell lines assessed by morphology.** Cells were exposed to H_2O_2 at increasing concentrations for 24 h, and slides were prepared using a cytospin. The slides were treated with Wright's stain and examined by light microscopy. Shown are the mean and S.E. of independent determinations on three separate experiments. Apoptosis of the HL-60 cells was significantly higher at 20 and 200 μM ($p = 0.0001$ and $p = 0.0002$, respectively, by one-way analysis of variance) compared with the K562 cells and spent HL-60 as indicated by *asterisks*.

activity and was capable of generating POBN/POBN$^{\cdot}$ when H_2O_2 and POBN were added (results not shown). We suspect that exogenous MPO fails to support H_2O_2-induced apoptosis, because it cannot reach the critical intracellular site to support the oxidative chemistry required for apoptosis. This suggests that the interaction of H_2O_2 with MPO occurs within the cell.

MPO Activity—The detection of the POBN$^{\cdot}$ suggested the possibility that MPO, which is known to be present in the HL-60 cells, is responsible for the POBN$^{\cdot}$, because earlier work had shown that its production is peroxidase-dependent in a cell-free system (37). Fig. 9*A* shows the comparative MPO activity of the three cell lines. The HL-60 line, which undergoes H_2O_2-induced apoptosis, contains appreciable activity that was significantly higher than that of the K562 cell line ($p = 0.003$). Most interesting was the observation that the spent HL-60 cells, which similar to the K562 cells fail to undergo apoptosis, contained only trace amounts of activity that was significantly less than that of the HL-60 cell line ($p = 0.00002$). This assay measures the activity of heme-peroxidase, and although optimized for MPO and eosinophil peroxidase, it is not specific for any one form of the enzyme (36). Therefore, we performed Western analysis using an antibody to MPO (39). The *inset* in Fig. 9*A* shows that the heavy subunit of MPO and precursor MPO are present in the HL-60 cells. No MPO protein was detected in the spent HL-60 or K562 cell lines.

Inhibition of Apoptosis by Methimazole—To further explore our hypothesis that MPO is essential for H_2O_2-induced apoptosis in the HL-60 cell, we used the antithyroid drug methimazole (1-methylimidazole-2-thiol) to inhibit MPO. First, studies on the concentration dependence of peroxidase inhibition by methimazole were carried out (Fig. 9*B*). Methimazole inhibited the MPO activity in the HL-60 line in a concentration-dependent fashion with full inhibition at 1 mM.

Next, we studied the effect of peroxidase inhibition on H_2O_2-induced apoptosis. HL-60 cells were incubated with 100, 250, 500, and 1000 μM methimazole, and then exposed to 20 μM H_2O_2 (Fig. 10). Methimazole alone did not induce apoptosis at any of the concentrations. When the effects of methimazole were studied using morphology to assess apoptosis, the results were confirmatory. H_2O_2-induced apoptosis in the HL-60 cells was inhibited by methimazole as estimated by morphology (0 μM, 34% of cells apoptotic; 0.1 μM, 8%; 0.25 μM, 2%; 0.5 μM, 1%; 1 μM, 0.7%). This demonstrates that methimazole inhibits H_2O_2-induced apoptosis in the HL-60 cell.

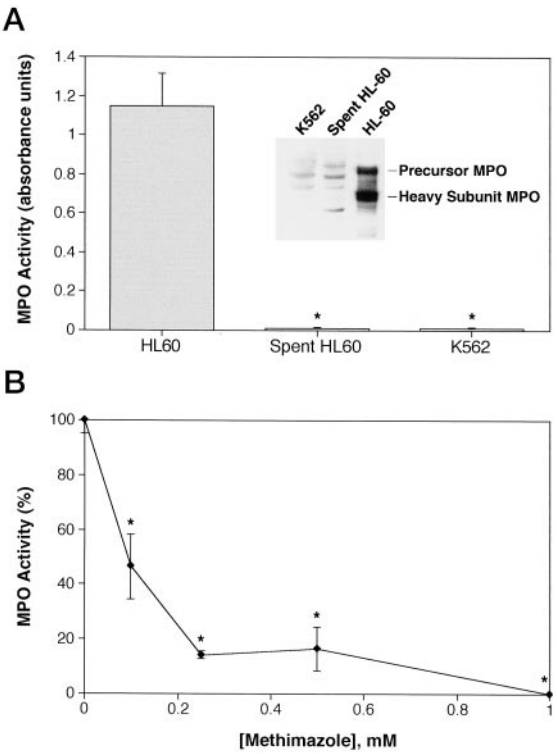

FIG. 9. **MPO activity of the cell lines and concentration dependence of the inhibitory effect of methimazole on MPO activity in the HL-60 cells.** *A*, MPO activity of the HL-60, K562, and spent HL-60 cell lines. MPO activity of the cells was determined by the ability to oxidize tetramethylbenzidine. The absorbance at 655 nm of cellular homogenates from 2×10^5 cells/ml was determined at 25 °C. The absorbance of the homogenates from the HL-60 cells was significantly higher than those from the spent HL-60 and K562 cells ($p = 0.003$) as indicated by the *asterisks*. *Inset*, MPO protein is expressed only in the HL-60 cell line. MPO protein expression of the three leukemia cell lines was measured using Western analysis. Monospecific rabbit polyclonal antiserum against MPO detected precursor MPO at 85–90 kDa and glycosylated heavy subunit at about 59 kDa only in the HL-60 cell line. *B*, concentration dependence of the inhibitory effect of methimazole on MPO activity of the HL-60 cells. Shown are the means and S.E. of triplicate determinations on three different samples. The overall difference in MPO activity across the levels of methimazole was highly significant ($p < 0.001$ by one-way analysis of variance). Dunnett's test showed that all pairwise comparisons *versus* control were significantly different as indicated by *asterisks*.

For confirmation, we also studied the effect of another heme inhibitor 4-aminobenzoic acid hydrazide. It inhibited H_2O_2-induced apoptosis across a broad range of concentrations from 50 μM to 1 mM (data not shown). At 10 mM, but not at lower concentrations, the inhibitor induced apoptosis in the absence of H_2O_2. In an activity assay, it inhibited MPO 93% at 50 μM and 100% at 100 μM.

Hypochlorous Acid Induces Apoptosis in Both HL-60 and Spent HL-60 Cells—We also carried out experiments using the derivative oxidant hypochlorous acid (HOCl). HL-60 cells were incubated with HOCl for 24 h and handled as in the H_2O_2 apoptosis experiments. We found that HOCl stimulates apoptosis in the HL-60 with a threshold of about 62 μM (Fig. 11*A*). These observations indicate that an oxidative substance generated by MPO induces apoptosis. Most interestingly, spent HL-60 cells, which fail to undergo apoptosis when exposed to H_2O_2, also responded to HOCl with apoptosis beginning at 93 μM (Fig. 11*B*).

DISCUSSION

MPO is a heme-protein that is abundant in the granules of many cells, including neutrophils, neutrophil precursors, and

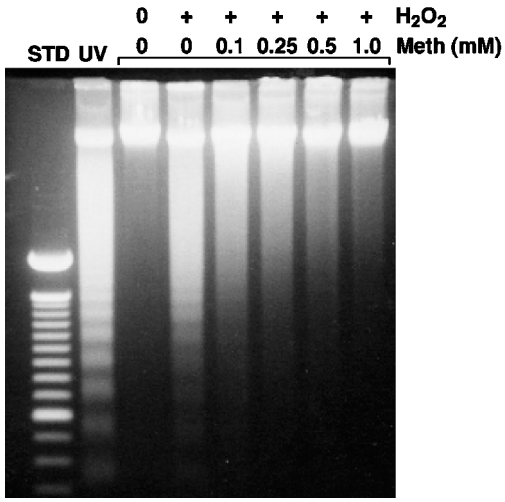

FIG. 10. **Inhibition of apoptosis by methimazole.** HL-60 cells were incubated with methimazole (*Meth*) for 10 min then treated with 20 μM H_2O_2 for 24 h. *Lanes* from *left* to *right* are DNA ladder standard, UV light-treated positive control, no methimazole and no H_2O_2, then H_2O_2 (20 μM) and increasing concentrations of methimazole. Shown is a representative gel from one of three independent experiments.

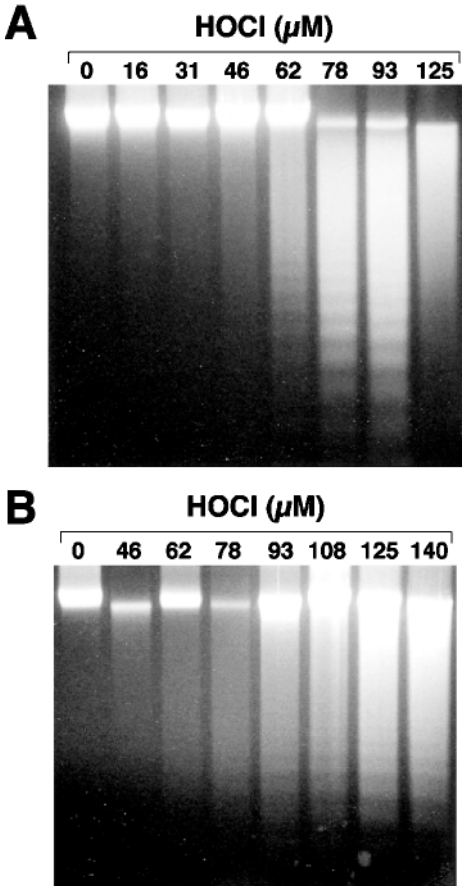

FIG. 11. **HOCl stimulates apoptosis in both HL-60 and spent HL-60 cells.** Cells were treated with HOCl for 24 h, and apoptosis was measured by DNA fragmentation. *A*, HOCl-induced apoptosis in HL-60 cells. Apoptosis is evident at 62 μM HOCl and higher. *B*, HOCl-induced apoptosis in spent HL-60 cells. Apoptosis is evident at 93 μM HOCl and higher.

macrophages, where its activity in the presence of H_2O_2 is important in the killing of ingested organisms (49–51). It is contained in the neutrophil primary azurophilic granules, which appear at the promyelocyte stage of myeloid maturation, and this fact explains its abundance in the HL-60 cell. Although one role is H_2O_2 removal, MPO uses H_2O_2 to oxidize a wide variety of substrates (52) resulting in the modification of lipids and proteins (53). However, a central role for peroxidase in conjunction with H_2O_2 in apoptosis had not been previously shown. The correlation of the presence of MPO in the HL-60 cell that undergoes apoptosis in response to H_2O_2, and its absence in the apoptotically silent corresponding K562 suggests a role for MPO. Most importantly, when the HL-60 cell is grown to high passage number and loses its MPO, it is no longer capable of responding to H_2O_2 to undergo apoptosis. In addition, inhibition of peroxidase activity in the HL-60 cell with methimazole eliminates apoptosis. Taken together this evidence strongly suggests that MPO has a central role in H_2O_2-induced apoptosis.

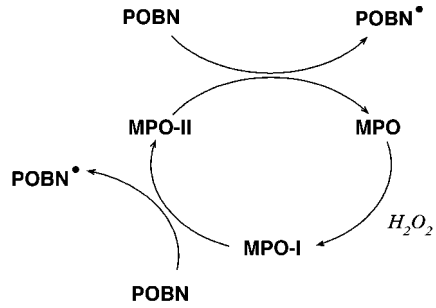

FIG. 12. **Reaction of H_2O_2 with MPO to generate MPO compounds I and II (MPO-I, MPO-II) and possible sites of generation of POBN$^\cdot$.**

The peroxidase-dependent production of POBN$^\cdot$ by the HL-60 cell during H_2O_2-induced apoptosis and the similarity of the H_2O_2 dose dependencies for apoptosis and radical generation (Figs. 1 and 5) are consistent with the possibility that the POBN$^\cdot$ is marking a peroxidase-mediated oxidative event leading to, or associated with, apoptosis induced by this oxidant. The lack of radical production by the K562 and spent HL-60 cell lines that do not contain MPO or undergo H_2O_2-induced apoptosis are compatible with that interpretation. The characteristic pattern of POBN$^\cdot$ generation in the two cell types is not due to a difference in antioxidant profile, because there is no difference in SOD or glutathione peroxidase and only small differences in catalase.

We postulate that H_2O_2 reacts with MPO leading to its reduction, generation of the MPO forms of compounds I and II (MPO-I, MPO-II), and a sequence of reductive events that generates POBN$^\cdot$ at one or both of two possible sites (Fig. 12). There may also be an unidentified oxidative intermediate such as tyrosine free radical (54) or another highly reactive product of H_2O_2/MPO. Any one of these, or another reactive product such as HOCl, NO_2^- (55), NO_2Cl (56), or chlorhydrin (57), could be the subsequent mediator of apoptosis. In any case, the evidence presented supports this chemistry rather than Fenton-type reactions.

Peroxidase and H_2O_2 are known to generate radical products from several redox-active compounds, including NADPH, glutathione, ascorbate, and trolox; the yield is amplified by the presence of NO_2^- (55). Lactoperoxidase combined with H_2O_2 forms a strong oxidant capable of converting NO_2^- to a strongly oxidizing metabolite (55), perhaps $^\cdot NO_2$ (58), and to oxidize melanin (59) and mitoxantrone (58). Similarly, MPO and H_2O_2 oxidize low density lipoprotein (21). The iron chelator deferoxamine reacts with MPO, lactoperoxidase, or horseradish peroxidase in the presence of H_2O_2 to form the deferoxamine radical, and POBN$^\cdot$ in the absence of deferoxamine at high concentrations of POBN (37). These studies were all done in cell-free systems. It

seems likely that, in our experiments with cells, POBN· is a product of the action of peroxidase/H_2O_2 by a similar reaction.

Methimazole is a known inhibitor of peroxidases, including MPO (60–63). Most work has been done on thyroid peroxidase, because methimazole is used clinically to treat hyperthyroidism. Oxidation of thyroid peroxidase by H_2O_2 produces an active radical heme-compound I that results in the iodination of thyroid hormone (62). Methimazole inactivates thyroid peroxidase reversibly or irreversibly depending upon conditions (61, 62). In our experiments, the inhibition of both POBN· formation and apoptosis by methimazole is consistent with compounds I and II being necessary species in these events.

The experiments with HOCl provide insight into the mechanism of the H_2O_2-induced apoptosis in HL-60 cells. They demonstrate that HOCl, a diffusible oxidant generated by MPO, is capable of directly inducing apoptosis in the HL-60 cells. Furthermore, HOCl induced apoptosis in the spent HL-60 cells, which do not undergo apoptosis in the presence of H_2O_2. These spent cells lack MPO activity, thus they are incapable of generating HOCl from H_2O_2. However HOCl added to the culture media is able to bypass the need for active MPO and induce apoptosis directly. This suggests that HOCl is the actual mediator of apoptosis.

Because H_2O_2 is a reactive oxidative substance, it has previously been assumed that its mechanism involves lipid peroxidation particularly as mediated by its metabolic product ·OH (64–67). Our observations, particularly at the concentrations inducing apoptosis, suggest that H_2O_2-mediated oxidative events at physiologically relevant concentrations may not necessarily be mediated primarily by Fenton chemistry. Oxidation by H_2O_2 leads to the formation of a profile of oxidative products quantitatively and qualitatively different from that of Fenton reagent ($H_2O_2 + Fe^{2+}$); therefore, the apoptosis we observed may have an oxidative basis other than lipid peroxidation. Only at the higher concentrations of H_2O_2, which induce necrosis, is there appreciable TBARS formation. It is possible that only the high concentrations of H_2O_2 that induce necrosis are mediated predominantly by Fenton chemistry reactions.

Our observations are important, because H_2O_2 is known to be a mediator of the toxicity of UV and ionizing radiation. UV-B produces H_2O_2, and there is evidence that H_2O_2 mediates the observed toxicity (6–8, 68). There are reports that the toxicity of ionizing radiation is due, at least in part, to H_2O_2 (10, 69–72). The best evidence for this is that catalase and glutathione peroxidase protect cells against its damaging effect (73–77). Similarly, H_2O_2 may mediate the toxicity of immunomodulators and chemotherapy (2, 14, 78–80). It is known that reactive oxygen species, including H_2O_2, can induce apoptosis in many cells types, including lymphocytes (81, 82), blastocysts (83), neutrophils (84), and HL-60 cells (85, 86). Jing *et al.* (87) showed that arsenic trioxide, a chemotherapeutic agent used to treat acute promyelocytic leukemia, induces apoptosis through an H_2O_2-dependent pathway. Tada-Oikawa *et al.* (80) showed that, during the apoptosis induced by the DNA-alkylating agent duocarmycin A, which is not a redox-cycling agent, the generation of a reactive oxygen species, possibly H_2O_2, precedes any change in mitochondrial potential and caspase activation in HL-60 cells. This suggests that an early oxidative event precedes any subsequent signaling event in the apoptotic process. If H_2O_2 mediates the toxicity of many important anticancer modalities, then our observations offer possible strategies for the therapeutic modulation of cellular oxidative balance and of therapies that have an oxidative component.

Acknowledgments—We thank Dr. William M. Nauseef for the antibody to MPO, Dr. Charles S. Davis for statistical assistance, Lori Manzel for advice on apoptosis gel techniques, and Drs. Bradley E. Britigan and Krysztof Reszka for helpful discussions.

REFERENCES

1. Meyer, M., Schreck, R., and Baeuerle, P. A. (1993) *EMBO J.* **12,** 2005–2015
2. Gotoh, Y., and Cooper, J. A. (1998) *J. Biol. Chem.* **273,** 17477–17482
3. Lee, S. F., Huang, Y. T., Wu, W. S., and Lin, J. K. (1996) *Free Radic. Biol. Med.* **21,** 437–448
4. Lennon, S. V., Martin, S. J., and Cotter, T. G. (1991) *Cell Prolif.* **24,** 203–214
5. Caceres-Dittmar, G., Ariizumi, K., Xu, S., Tapia, F. J., Bergstresser, P. R., and Takashima, A. (1995) *Photochem. Photobiol.* **62,** 176–183
6. Sato, K., Taguchi, H., Maeda, T., Minami, H., Asada, Y., Watanabe, Y., and Yoshikawa, K. (1995) *J. Invest. Dermatol.* **105,** 608–612
7. Bertling, C. J., Lin, F., and Girotti, A. W. (1996) *Photochem. Photobiol.* **64,** 137–142
8. Peus, D., Vasa, R. A., Meves, A., Pott, M., Beyerle, A., Squillace, K., and Pittelkow, M. R. (1998) *J. Invest. Dermatol.* **110,** 966–971
9. Otero, G., Avila, M. A., de la Pena, L., Emfietzoglou, D., Cansado, J., Popescu, G. F., and Notario, V. (1997) *Carcinogenesis* **18,** 1569–1575
10. Saran, M., and Bors, W. (1997) *Radiat. Res.* **147,** 70–77
11. Saran, M., Winkler, K., and Fellerhoff, B. (1997) *Int. J. Radiat. Biol.* **72,** 745–750
12. Buettner, G. R., and Need, M. J. (1985) *Cancer Lett.* **25,** 297–304
13. Tuttle, S. W., Hazard, L., Koch, C. J., Mitchell, J. B., Coleman, C. N., and Biaglow, J. E. (1994) *Int. J. Radiat. Oncol. Biol. Phys.* **29,** 357–362
14. Komiyama, T., Kikuchi, T., and Sugiura, Y. (1986) *J Pharmacobio-dyn.* **9,** 651–664
15. Arrick, B. A., Griffo, W., Cohn, Z., and Nathan, C. (1985) *J. Clin. Invest.* **76,** 567–574
16. Puppo, A., and Halliwell, B. (1988) *Biochem. J.* **249,** 185–190
17. Mello Filho, A. C., Hoffmann, M. E., and Meneghini, R. (1984) *Biochem. J.* **218,** 273–275
18. Qian, S. Y., and Buettner, G. R. (1999) *Free Radic. Biol. Med.* **26,** 1447–1456
19. Christ, M., Luu, B., Mejia, J. E., Moosbrugger, I., and Bischoff, P. (1993) *Immunology* **78,** 455–460
20. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) *Blood* **92,** 3007–3017
21. Hazen, S. L., and Heinecke, J. W. (1997) *J. Clin. Invest.* **99,** 2075–2081
22. Lamb, N. J., Gutteridge, J. M., Baker, C., Evans, T. W., and Quinlan, G. J. (1999) *Crit. Care Med.* **27,** 1738–1744
23. Kroemer, G., Dallaporta, B., and Resche-Rigon, M. (1998) *Annu. Rev. Physiol.* **60,** 619–642
24. Reed, J. C. (1999) *J. Clin. Oncol.* **17,** 2941–2953
25. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309–1312
26. Chau, Y. P., Shiah, S. G., Don, M. J., and Kuo, M. L. (1998) *Free Radic. Biol. Med.* **24,** 660–670
27. Kakeya, H., Onose, R., and Osada, H. (1998) *Cancer Res.* **58,** 4888–4894
28. Fabisiak, J. P., Tyurina, Y. Y., Tyurin, V. A., Lazo, J. S., and Kagan, V. E. (1998) *Biochemistry* **37,** 13781–13790
29. Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J. L., Petit, P. X., and Kroemer, G. (1995) *J. Exp. Med.* **181,** 1661–1672
30. Gallagher, R., Collins, S., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F., and Gallo, R. (1979) *Blood* **54,** 713–733
31. Nauseef, W. M. (1986) *Blood* **67,** 865–872
32. Mansat, V., Laurent, G., Levade, T., Bettaieb, A., and Jaffrezou, J. P. (1997) *Cancer Res.* **57,** 5300–5304
33. Lozzio, B. B., and Lozzio, C. B. (1979) *Leukocyte Res.* **3,** 363–370
34. Wagner, B. A., Buettner, G. R., Oberley, L. W., and Burns, C. P. (1998) *Cancer Res.* **58,** 2809–2816
35. Kravtsov, V. D., Greer, J. P., Whitlock, J. A., and Koury, M. J. (1998) *Blood* **92,** 968–980
36. Bozeman, P. M., Learn, D. B., and Thomas, E. L. (1990) *J Immunol. Methods* **126,** 125–133
37. McCormick, M. L., Buettner, G. R., and Britigan, B. E. (1995) *J. Biol. Chem.* **270,** 29265–29269
38. Laemmli, U. K. (1970) *Nature* **227,** 680–685
39. Nauseef, W. M., Root, R. K., and Malech, H. L. (1983) *J. Clin. Invest.* **71,** 1297–1307
40. Buettner, G. R. (1990) *Free Radic. Res. Commun.* **10,** 5–9
41. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
42. Spitz, D. R., and Oberley, L. W. (1989) *Anal. Biochem.* **179,** 8–18
43. Aebi, H. (1984) *Methods Enzymol.* **105,** 121–126
44. Lawrence, R. A., and Burk, R. F. (1976) *Biochem. Biophys. Res. Commun.* **71,** 952–958
45. Dunnett, C. W. (1955) *J. Am. Stat. Assoc.* **50,** 1096–1121
46. Kostka, P., and Kwan, C.-Y. (1989) *Lipids* **24,** 545–549
47. North, J. A., Spector, A. A., and Buettner, G. R. (1992) *J. Biol. Chem.* **267,** 5743–5746
48. Wagner, B. A., Buettner, G. R., and Burns, C. P. (1993) *Cancer Res.* **53,** 711–713
49. Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) *J. Clin. Invest.* **94,** 437–444
50. Nauseef, W. M. (1988) *Hematol Oncol. Clin. North Am.* **2,** 135–158
51. Nauseef, W. M. (1998) *J. Mol. Med.* **76,** 661–668
52. Halliwell, B., and Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, 2nd. Ed., pp. 86–123, Clarendon Press, Oxford
53. Hazen, S. L., Hsu, F. F., Gaut, J. P., Crowley, J. R., and Heinecke, J. W. (1999) *Methods Enzymol.* **300,** 88–105
54. Heinecke, J. W. (1999) *J. Lab. Clin. Med.* **133,** 321–325
55. Reszka, K. J., Matuszak, Z., Chignell, C. F., and Dillon, J. (1999) *Free Radic. Biol. Med.* **26,** 669–678

56. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) *Nature* **391,** 393–397
57. Winterbourn, C. C., van den Berg, J. J., Roitman, E., and Kuypers, F. A. (1992) *Arch. Biochem. Biophys.* **296,** 547–555
58. Reszka, K. J., Matuszak, Z., and Chignell, C. F. (1997) *Chem. Res. Toxicol.* **10,** 1325–1330
59. Reszka, K. J., Matuszak, Z., and Chignell, C. F. (1998) *Free Radic. Biol. Med.* **25,** 208–216
60. Bandyopadhyay, U., Bhattacharyya, D. K., and Banerjee, R. K. (1993) *Biochem. J.* **296,** 79–84
61. Taurog, A., Dorris, M. L., and Guziec, F. S., Jr. (1989) *Endocrinology* **124,** 30–39
62. Doerge, D. R., and Divi, R. L. (1995) *Xenobiotica* **25,** 761–767
63. Pincemail, J., Deby, C., Thirion, A., de Bruyn-Dister, M., and Goutier, R. (1988) *Experientia (Basel)* **44,** 450–453
64. Lomonosova, E. E., Kirsch, M., and de Groot, H. (1998) *Free Radic. Biol. Med.* **25,** 493–503
65. Farber, J. L., Kyle, M. E., and Coleman, J. B. (1990) *Lab. Invest.* **62,** 670–679
66. Coyle, J. T., and Puttfarcken, P. (1993) *Science* **262,** 689–695
67. Halliwell, B., and Aruoma, O. I. (1991) *FEBS Lett.* **281,** 9–19
68. Reszka, K., Kolodziejczyk, P., Tsoungas, P. G., and Lown, J. W. (1988) *Photochem. Photobiol.* **47,** 625–633
69. Robinson, S., Bevan, R., Lunec, J., and Griffiths, H. (1998) *FEBS Lett.* **430,** 297–300
70. Gallin, E. K., and Green, S. W. (1987) *Blood* **70,** 694–701
71. Meneghini, R., and Hoffmann, M. E. (1980) *Biochim. Biophys. Acta* **608,** 167–173
72. Croute, F., Soleilhavoup, J. P., Vidal, S., Dupouy, D., and Planel, H. (1982) *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **41,** 209–214
73. Yamada, K., Ito, A., Watanabe, H., Takahashi, T., Basaran, N. H., and Gotoh, T. (1997) *Anticancer Res.* **17,** 2041–2047
74. Krinsky, N. I., Sladdin, D. G., Levine, P. H., Taub, I. A., and Simic, M. G. (1981) *Thromb. Haemostasis* **45,** 116–120
75. Smith, S. T., and Claycamp, H. G. (1988) *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* **53,** 829–837
76. Sohal, R. S., Ku, H. H., and Agarwal, S. (1993) *Biochem. Biophys. Res. Commun.* **196,** 7–11
77. Borek, C., Ong, A., Mason, H., Donahue, L., and Biaglow, J. E. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1490–1494
78. Morikawa, K., Kamegaya, S., Yamazaki, M., and Mizuno, D. (1985) *Cancer Res.* **45,** 3482–3486
79. Sangeetha, P., Das, U. N., Koratkar, R., and Suryaprabha, P. (1990) *Free Radic. Biol. Med.* **8,** 15–19
80. Tada-Oikawa, S., Oikawa, S., Kawanishi, M., Yamada, M., and Kawanishi, S. (1999) *FEBS Lett.* **442,** 65–69
81. Hansson, M., Asea, A., Ersson, U., Hermodsson, S., and Hellstrand, K. (1996) *J. Immunol.* **156,** 42–47
82. Kasai, T., Ohguchi, K., Nakashima, S., Ito, Y., Naganawa, T., Kondo, N., and Nozawa, Y. (1998) *J. Immunol.* **161,** 6469–6474
83. Pierce, G. B., Parchment, R. E., and Lewellyn, A. L. (1991) *Differentiation* **46,** 181–186
84. Lundqvist-Gustafsson, H., and Bengtsson, T. (1999) *J. Leukocyte Biol.* **65,** 196–204
85. Witenberg, B., Kalir, H. H., Raviv, Z., Kletter, Y., Kravtsov, V., and Fabian, I. (1999) *Biochem. Pharmacol.* **57,** 823–832
86. DiPietrantonio, A. M., Hsieh, T., and Wu, J. M. (1999) *Biochem. Biophys. Res. Commun.* **255,** 477–482
87. Jing, Y., Dai, J., Chalmers-Redman, R. M., Tatton, W. G., and Waxman, S. (1999) *Blood* **94,** 2102–2111