

Oxidative Stress Inhibits Apoptosis in Human Lymphoma Cells*

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Apoptosis and necrosis are two forms of cell death that are induced under different conditions and that differ in morphological and biochemical features. In this report, we show that, in the presence of oxidative stress, human B lymphoma cells are unable to undergo apoptosis and die instead by a form of necrosis. This was established using the chemotherapy drug VP-16 or the calcium ionophore A23187 to induce apoptosis in Burkitt's lymphoma cell lines and by measuring classical markers of apoptotic death, including cell morphology, annexin V binding, DNA ladder formation, and caspase activation. In the presence of relatively low levels of H₂O₂ (75–100 μ M), VP-16 and A23187 were unable to induce apoptosis in these cells. Instead, the cells underwent non-apoptotic cell death with mild cytoplasmic swelling and nuclear shrinkage, similar to the death observed when they were treated with H₂O₂ alone. We found that H₂O₂ inhibits apoptosis by depleting the cells of ATP. The effects of H₂O₂ can be overcome by inhibitors of poly(ADP)-ribosylation, which also preserve cellular ATP levels, and can be mimicked by agents such as oligomycin, which inhibit ATP synthesis. The results show that oxidants can manipulate cell death pathways, diverting the cell away from apoptosis. The potential physiological ramifications of this finding will be discussed.

Cell death can occur through several different mechanisms, which are distinguished by unique morphological and biochemical traits and which have distinct physiological ramifications. The two most widely described forms of cell death are necrosis and apoptosis (reviewed in Ref. 1). Necrosis is induced by severe environmental disturbances and is characterized by swelling of the cytoplasm and cytoplasmic organelles, early rupture of the plasma membrane, clumping of the chromatin, and usually swelling of the nucleus (1). Apoptosis is regarded as an active and progressive response to physiologic and pathologic stimuli (2, 3). It is characterized by early and prominent condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of proteases and endonucleases, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the cells into membrane-bound "apoptotic bodies." A significant physiological consequence of cell death by apoptosis is that the apoptotic bodies can be phagocytosed by nearby cells such that the contents are degraded intracellularly (4). As a result, cells dying by apoptosis cause minimal disturbance

to the surrounding tissue. In contrast, the rupture of necrotic cells and the release of lysosomal and other enzymes into the surrounding tissue causes further tissue destruction and inflammation (1).

This report examines the effects of oxidative stress on the cell death machinery. Oxidants such as superoxide, hydrogen peroxide (H₂O₂), and the hydroxyl radical are generated under a variety of conditions *in vivo* such as during acute and chronic inflammation (5). Treatment of cells *in vitro* with H₂O₂ causes DNA strand breaks (6, 7), oxidation of lipids (8) and proteins (9), activation of poly(ADP)-ribosylation (10), and depletion of cellular energy stores (6, 11). Depending on the concentration of H₂O₂ employed and the type of cell being studied, the mode of cell death induced by H₂O₂ has been reported to be either apoptosis or necrosis (12–14), with necrosis generally being reported with higher concentrations of the oxidant (12, 15).

In a previous study, we found that Burkitt's lymphoma cells were highly susceptible to killing by H₂O₂ and that overexpression of the *bcl-2* oncogene, which is known to inhibit apoptosis (16), did not prevent H₂O₂-induced cell death (14), contrary to earlier studies suggesting that *bcl-2* protects cells from oxidant-induced killing (17). It was concluded that *bcl-2* was unable to inhibit the cell killing by H₂O₂ because the primary form of cell death induced was non-apoptotic. In the present studies, we investigated the effects of H₂O₂ on the cell death machinery and found that H₂O₂ actually inhibits induction of apoptosis in Burkitt's lymphoma cells. That is, in the presence of H₂O₂, agents that normally kill Burkitt's lymphoma cells by inducing apoptosis are no longer able to do so. H₂O₂ inhibited all the major steps of apoptosis. The effects of H₂O₂ can be explained by its ability to deplete the cells of ATP. Thus, oxidative stress can manipulate the mechanism of cell death, diverting it away from apoptosis to necrosis.

MATERIALS AND METHODS

Cells—The Burkitt's lymphoma cell lines JLP 119, ST-486, and BL-41 were provided by Kishor Bhatia from the laboratory of Ian Magrath (NCI, National Institutes of Health, Bethesda, MD). Cells were grown in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 μ M β -mercaptoethanol at 37 °C in 5% CO₂ in air.

Cell Treatments—Exponentially growing cells were harvested by centrifugation and resuspended in fresh media to achieve a culture density of 5×10^5 cells/ml. H₂O₂ was added to the cell suspensions at the beginning of the experiments or after a 30-min preincubation with VP-16. The cells were then incubated for 2–24 h as indicated in the text. The poly (ADP-ribose) polymerase (PARP)¹ inhibitors 3-aminobenzamide (3AB) and 4-hydroxyquinazoline (4HQ) were added to cell suspensions 30 min prior to H₂O₂. For the experiments with oligomycin, cells were washed and resuspended in RPMI 1640 medium without glucose but supplemented with 10% fetal calf serum, 2 mM L-glutamine,

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¹ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; 3AB, 3-aminobenzamide; 4HQ, 4-hydroxyquinazoline; PI, propidium iodide; FITC, fluorescein isothiocyanate; AFC, 7-amino-4-trifluoromethyl coumarin; FACS, fluorescence-activated cell sorter; Cbz, carbobenzoxy; GDI, GDP dissociation inhibitor.

and 50 μM β -mercaptoethanol. After adaptation to this medium for 30 min, cells were exposed to 10 μM oligomycin.

Morphological Assessment of Apoptosis Using Hoechst/Propidium Iodide Nuclear Staining and Fluorescence Microscopy—Cells were stained with Hoechst 33342 and propidium iodide (PI) and visualized using fluorescence microscopy as described previously (14). A minimum of 200 cells were counted and classified as follows: (i) live cells (normal nuclei: blue chromatin with organized structure); (ii) membrane-intact apoptotic cells (bright blue chromatin, which is highly condensed, marginated, or fragmented); (iii) necrotic cells (red, enlarged nuclei with smooth normal structure); (iv) membrane-permeable apoptotic cells (bright red chromatin, highly condensed or fragmented); (v) pyknotic/necrotic cells (dense, bright red, slightly condensed nuclei sometimes divided into two or three spheres).

Conventional Agarose Gel Electrophoresis for Detection of Nucleosomal DNA Fragmentation—Total cellular DNA was extracted by the procedure of Smith *et al.* (18). DNA samples (equivalent to $\sim 2 \times 10^6$ cells) were applied to 2% agarose gels (molecular grade agarose, Life Technologies, Inc.), and electrophoresis was performed using 1 \times TBE for 12–15 h at 30 V. *Hae*III-digested ϕ X174 (New England Biolabs, Beverly, MA) was used as a molecular weight standard.

Annexin V Binding Assay—The apoptosis detection kit (catalog no. KNX50) from R&D Systems was used according to the manufacturer's instructions. Cells were analyzed (10,000 cells/sample) on a FACScan (Becton Dickinson, San Jose, CA) using CELLQUEST flow cytometric analysis software. FITC was detected using a 530/30-nm bandpass filter (FL1 channel), and PI was measured at 610 nm (FL2 channel). Cross-over of FITC fluorescence into the PI detection window was electronically compensated.

Measurement of Caspase Activities—The activities of CPP32 (caspase-3) and other caspases were measured as described by others with minor modifications (19). Briefly, cells treated variously were collected by centrifugation, washed twice with phosphate-buffered saline without Ca^{2+} or Mg^{2+} , and lysed in ICE buffer (50 mM Hepes buffer, pH 7.5, 10% sucrose, and 0.1% Triton X-100) at a concentration of 10^7 cells/ml for 20 min on ice. After centrifugation at $10,000 \times g$ for 10 min at 4 °C, supernatants were transferred to a tube containing dithiothreitol at a final concentration of 10 mM. 7-Amino-4-trifluoromethyl coumarin (AFC)-conjugated peptide substrate for each caspase was added to the cell lysates (75 μl , 1.5×10^6 cells) to a final concentration of 50 μM , and the final reaction volume was adjusted to 200 μl with ICE buffer. After incubation at 30 °C for 1 h, reactions were stopped by adding 2 ml of ice-cold phosphate-buffered saline without Ca^{2+} or Mg^{2+} to each tube and the levels of released AFC were measured using a spectrofluorometer (PTI Delta Scan 1, Photon Technology International, Monmouth Junction, NJ) with excitation at 400 nm and emission at 505 nm. A 10 μM solution of free AFC gives about 10×10^4 fluorescence units in this system. The specific substrates for each caspase were as follows: carbobenzoxy (Cbz)-Tyr-Val-Ala-Asp-AFC (z-YVAD-AFC) for caspase-1, Cbz-Val-Asp-Val-Ala-Asp-AFC (z-VDVAD-AFC) for caspase-2, Cbz-Asp-Glu-Val-Ala-Asp-AFC (z-DEVD-AFC) for caspases-3 and -7, Cbz-Val-Glu-Ile-Asp-AFC (z-VEID-AFC) for caspase-6, and Cbz-Ile-Glu-Thr-Asp-AFC (z-IETD-AFC) for caspase-8. The substrates were all dissolved in Me_2SO and stored at -20 °C.

Western Blot Analysis—Total cell lysates were prepared, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to membranes as described previously (14). After blocking with 5% milk, membranes were incubated with rabbit polyclonal anti-bovine PARP, mouse monoclonal anti-human CPP32, rabbit polyclonal anti-human DNA-PKcs, mouse monoclonal anti-human NuMA, rabbit polyclonal anti-human Rho-GDI/D4-GDI, mouse monoclonal anti-human ICH-1, or goat polyclonal anti-human lamin B, followed by a 1-h incubation with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized by chemiluminescence using the ECL kit from NEN Life Science Products.

ATP Assay—Intracellular ATP levels were determined using luciferin-luciferase (20). Briefly, cells ($\sim 5 \times 10^5$ cells) that had been treated with H_2O_2 or oligomycin were collected by centrifugation; resuspended in 250 μl of 10 mM KH_2PO_4 , 4 mM MgSO_4 , pH 7.4; heated at 98 °C for 4 min; and placed on ice. At the time of the assay, a 50- μl sample ($\sim 1 \times 10^5$ cells) was added to 100 μl of 50 mM NaAsO_2 , 20 mM MgSO_4 , pH 7.4, and 80 μg of luciferin/luciferase. Light emission was quantified in a Dynatech ML 3000 microtiter plate luminometer (Chantilly, VA). ATP standard curves were run in all experiments and were linear in the range of 5–2500 nM. Stock ATP concentrations were measured spectrophotometrically at 259 nm using an extinction coefficient of 15,400.

Reagents—VP-16, 3-AB, 4-HQ, oligomycin, A23187, and luciferin-luciferase were purchased from Sigma. AFC-labeled caspase substrates

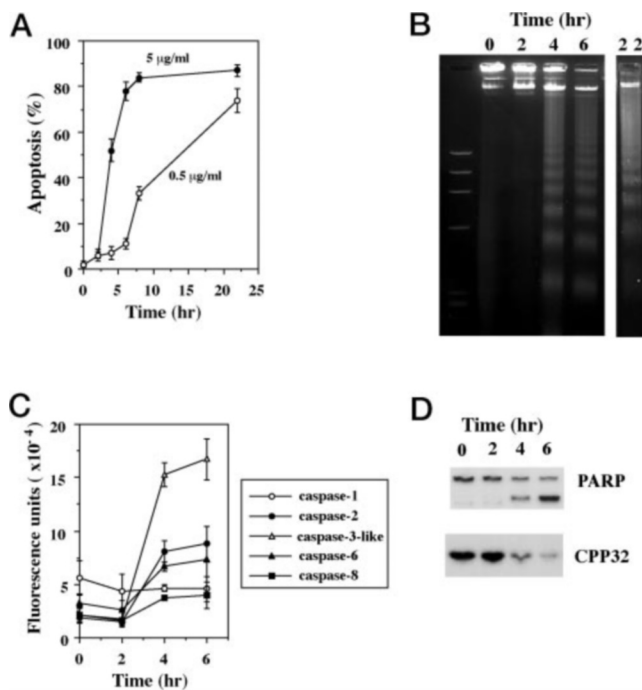


FIG. 1. VP-16 induces classical apoptosis in JLP 119 cells. JLP 119 cells were treated with VP-16 at a concentration of 5 $\mu\text{g/ml}$ for 0–6 h or at a concentration of 0.5 $\mu\text{g/ml}$ for 0–24 h. Cells were collected at the times indicated and assayed apoptosis as follows. **A**, morphological assay using Hoechst/PI staining and fluorescence microscopy (mean \pm range, $n = 2$). **B**, DNA ladder formation by conventional agarose gel electrophoresis. The gel samples reflecting 2-, 4-, and 6-h time points are from cells treated with 5 $\mu\text{g/ml}$ VP-16, and the sample at 22 h is from cells treated with 0.5 $\mu\text{g/ml}$ VP-16. **C**, Activation of caspase activities in total cell lysates using AFC-conjugated substrates specific for each enzyme (mean \pm std, $n = 3$). "Caspase-3-like" refers to all DEVDase activities. **D**, cleavage of the intracellular proteins PARP and CPP32 by Western blot analysis. All samples in **C** and **D** are from cells treated with 5 $\mu\text{g/ml}$ VP-16. The data shown in **B** and **D** are from representative studies that were repeated at least three times.

were from Enzyme Systems Products (Dublin, CA). Antibodies to intracellular proteins were from Biomol (Plymouth Meeting, PA) for PARP, Transduction Laboratories (Lexington, KY) for CPP32, Oncogene Sciences (Cambridge, MA) for DNA-PK and NuMA, Pharmingen (San Diego, CA) for ICH-1 and GDI/D4-GDI, and Santa Cruz Biotechnology Inc. (Santa Cruz, CA) for Lamin B. Horseradish peroxidase-conjugated secondary antibodies were from Southern Biotechnologies (Birmingham, AL).

RESULTS

Modes of Cell Death Induced in Burkitt's Lymphoma Cells by VP-16 and H_2O_2 —VP-16 (etoposide) is a topoisomerase II inhibitor, which is widely used for cancer chemotherapy (21) and is known to kill a variety of different tumor cells by inducing apoptosis. As shown in Fig. 1, VP-16 kills JLP 119 Burkitt's lymphoma cells entirely by inducing apoptosis. The time course of cell killing varies depending on the concentration of drug employed. Thus, at low concentrations of drug (0.5 $\mu\text{g/ml}$), induction of apoptosis in 75% of the cells (as determined by fluorescence microscopy) takes place gradually over the course of a 22-h incubation (Fig. 1A). By comparison, 80% of the cells show morphological signs of apoptosis within 6 h when treated with 5 $\mu\text{g/ml}$ VP-16. VP-16-treated cells also show other classical markers of apoptosis (shown in Fig. 1, B–D, for the 5 $\mu\text{g/ml}$ treatment) such as oligonucleosomal degradation of the DNA (ladder formation); cleavage of the caspase-3 protein (CPP32); activation of the enzyme activities of caspases-2, -3, and -6; and cleavage of poly(ADP-ribose) polymerase (PARP). Note that the substrate for assaying caspase-3 activity (DEVD) can also be cleaved by caspase-7 (22), and possibly caspases-8 and 10 (23).

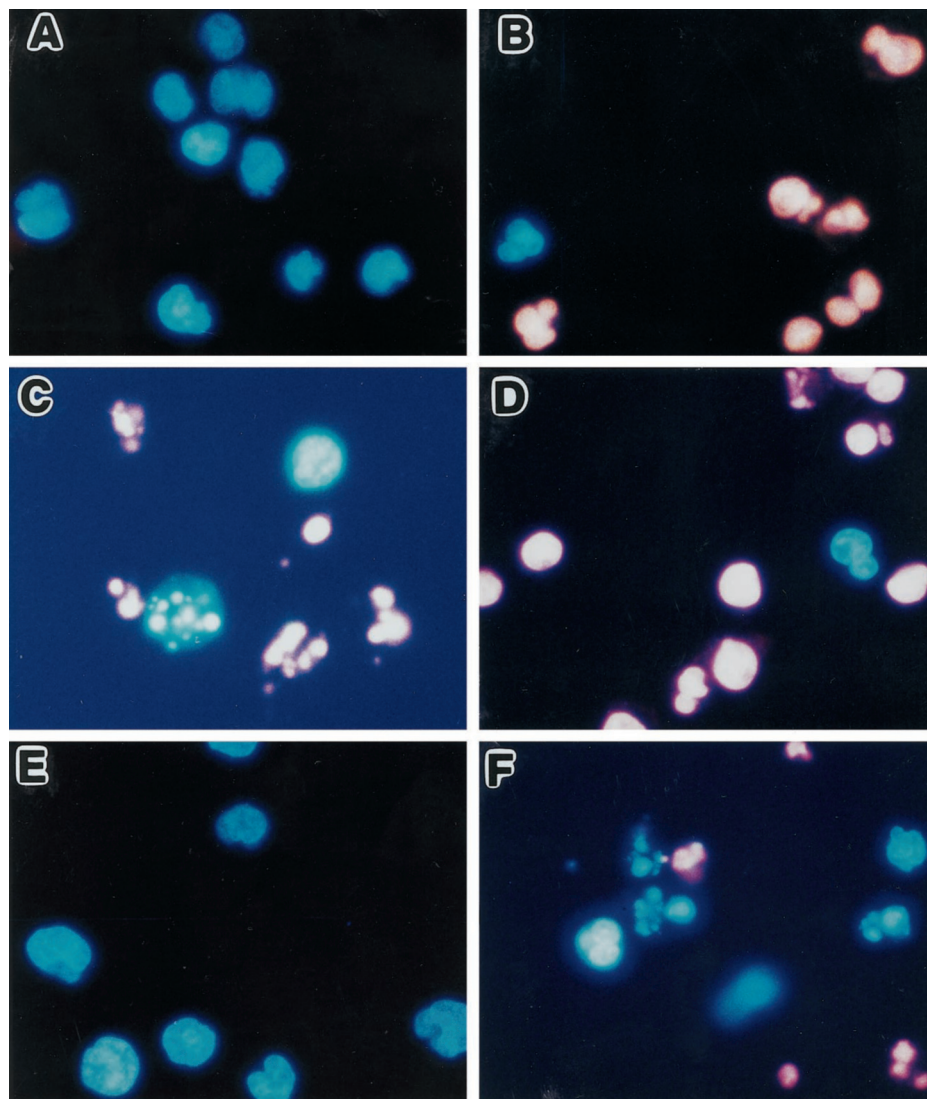


FIG. 2. **Morphology of cells treated with H_2O_2 , VP-16, and 3AB.** JLP 119 cells were treated for 22 h, harvested, stained with Hoechst/PI, and examined by fluorescence microscopy as described under "Materials and Methods" (original magnification, $\times 400$). Cells were treated with: A, no treatment; B, H_2O_2 , 75 μM ; C, VP-16, 1 $\mu g/ml$; D, H_2O_2 and VP-16; E, 3AB, 0.5 mM; F, H_2O_2 and 3AB.

Hence, the activity displayed with this substrate is referred to as "caspase-3-like."

In contrast to VP-16, when Burkitt's lymphoma cells are treated with 75–100 μM H_2O_2 , the predominant form of cell death induced is non-apoptotic. This was determined previously by measuring molecular DNA fragmentation by conventional agarose gel electrophoresis and a FACS terminal deoxynucleotidyltransferase-mediated dUTP-x nick end labeling assay (14). Examination of cell morphology by fluorescence microscopy shows that most of the cells treated with 75–100 μM H_2O_2 remain roughly the same size as untreated cells and the nucleus undergoes mild nuclear condensation (pyknosis) without fragmentation. Examples of the morphological changes induced by H_2O_2 can be seen in Fig. 2, where *panel A* shows control (untreated) cells and *panel B* shows cells treated with 75 μM H_2O_2 . Because these cells bear none of the classical biochemical or morphological features of apoptosis (14) and see below) yet show smaller nuclei than classical necrotic cells, we refer to them as pyknotic/necrotic. The data shown are for the Burkitt's lymphoma cell line JLP 119. Identical results were obtained with two other Burkitt's lymphoma cell lines: ST-486 and BL-41.

H_2O_2 Inhibits the Ability of VP-16 and A23187 to Induce Apoptosis—To gain insight into the possible effects of oxidative stress on cell death pathways, we tested the effect of H_2O_2 on VP-16-induced apoptosis. In these experiments, 75 μM H_2O_2

was added to the cells 30 min after adding VP-16 (0.5 $\mu g/ml$) and the cells were allowed to incubate overnight. The mode of cell death was determined by fluorescence microscopy using Hoechst/PI staining. Photographs showing representative cell morphologies are given in Fig. 2, and quantitative results are shown in Fig. 3A. The induction of apoptosis by 1 $\mu g/ml$ VP-16 (Fig. 2C) was almost completely inhibited by addition of 75 μM H_2O_2 (Fig. 2D) and the cells died instead by pyknosis/necrosis, identical to the mode of cell death induced by H_2O_2 alone (Fig. 2B). In control experiments, H_2O_2 did not interact with and inactivate VP-16 directly. This was determined by preincubating VP-16 with 100 μM H_2O_2 for 30 min, removing the H_2O_2 with catalase, and then adding the VP-16 to the cells. Full apoptosis inducing activity was maintained. In addition, direct treatment with H_2O_2 did not alter the absorption spectrum of VP-16. The ability of H_2O_2 to inhibit VP-16-induced apoptosis was confirmed using two other Burkitt's lymphoma cell lines, ST-486 and BL-41 (data not shown).

The effects of H_2O_2 on VP-16-induced cell killing could be overcome by co-treating the cells with two inhibitors of poly-(ADP-ribose) polymerase (PARP). These were 3AB (0.5 mM, Fig. 2F) and 4HQ (50 μM , data not shown). H_2O_2 also inhibited induction of DNA ladder formation by VP-16 and this inhibition was also overcome by 3AB (Fig. 3B) and 4HQ (data not shown). The concentrations of 3AB and 4HQ employed in these experiments are expected to cause specific inhibition of PARP

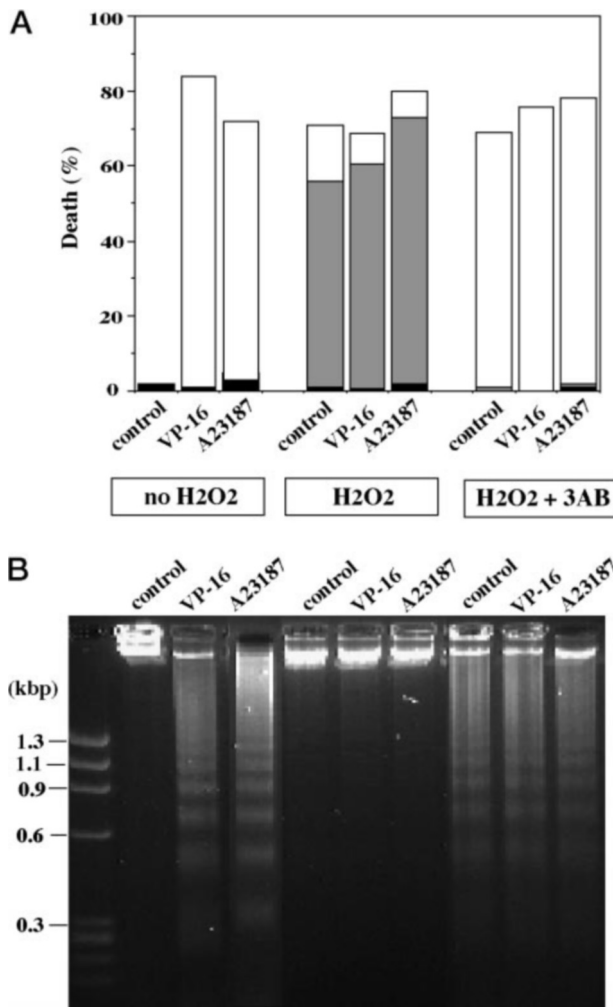


FIG. 3. Effects of H_2O_2 and 3AB on induction of apoptosis by VP-16 or A23187. JLP 119 cells were incubated with VP-16 (1 μ g/ml) or A23187 (1 μ g/ml) in the absence or presence of H_2O_2 (75 μ M) or H_2O_2 /3AB (0.5 mM) for 22 h (VP-16) or 48 h (A23187). **A**, quantitative assessment of cell viability and morphology by fluorescence microscopy. The results represent the mean of three separate experiments. □, apoptotic; ▤, pyknotic/necrotic; ■, necrotic. **B**, analysis of DNA fragmentation by conventional agarose gel electrophoresis. The data are from a representative study that was repeated at least three times.

without affecting mono(ADP-ribosyl)ation reactions (24). Neither compound caused any toxicity to the cells by themselves (Figs. 2E and 3A). Both PARP inhibitors also converted the mode of cell death induced by H_2O_2 alone from pyknosis/necrosis to apoptosis (Fig. 2F and Fig. 3; data shown for 3AB only). Identical results were obtained when the calcium ionophore A23187 was employed to induce apoptosis instead of VP-16 (Fig. 3). A23187-induced apoptosis was inhibited by addition of 75 μ M H_2O_2 , and this effect of H_2O_2 was overcome by inhibitors of PARP.

A concentration-response study of cell death induced by H_2O_2 alone is shown in Fig. 4. Note that, at most, only 20% of the cells die by apoptosis and this occurs at a narrow concentration range around 50 μ M. At higher concentrations (75–100 μ M), the predominant form of cell death induced is pyknosis/necrosis.

One of the earliest markers of apoptosis is the translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane (25). This change can be detected by testing for binding of the protein annexin V to the cell surface (26). To determine whether cells undergo this early apoptotic change in the presence of H_2O_2 , ST-486 cells were treated with VP-16, H_2O_2 , or both for various times and annexin V binding was

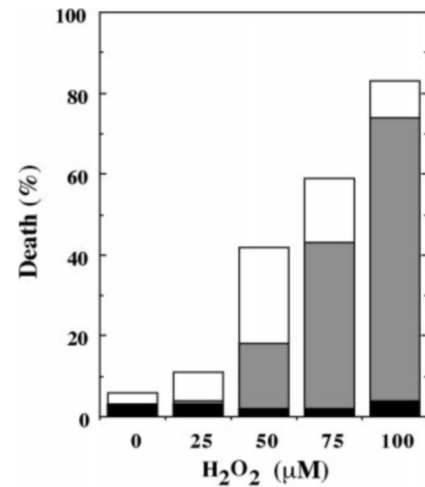


FIG. 4. Concentration-response study of H_2O_2 -induced cell death. Cells were treated with the indicated concentrations of H_2O_2 and incubated for approximately 21 h at 37 °C. Cell death was measured by fluorescence microscopy using Hoechst/PI staining to examine nuclear morphologies. □, apoptotic; ▤, pyknotic/necrotic; ■, necrotic.

measured by FACS analysis. ST-486 cells were employed in place of JLP 119 cells because the latter had unusually high control levels of annexin V staining which interfered with quantification of VP-16-induced staining. As shown in Fig. 5, VP-16 (30 μ g/ml) induced a 4–5-fold induction of annexin V binding over the course of a 6-h incubation, during which time roughly 60–70% of the cells developed morphological features of apoptosis. H_2O_2 inhibited this increase by 50–60% and did not by itself induce any increase in annexin V staining.

Another hallmark feature of apoptosis inhibited by H_2O_2 is the activation of caspases (reviewed in Ref. 22). In the following experiments, different caspase activities were measured by incubating cell extracts from treated and control cells with specific peptide substrates for each enzyme. As shown in Fig. 6, caspase-1 activity was constitutively expressed in JLP 119 cells and was not induced further by VP-16 treatment. Caspases-2, -3, -6, and -8 were all induced by VP-16 treatment, with caspase-3-like (DEVDase) activities being the most profoundly activated. Co-treatment of the cells with H_2O_2 prevented this activation in all cases. Inhibition of the activation of caspase activity occurred only when H_2O_2 was added to cells and not when it was added to cell extracts, indicating that the oxidant inhibits activation of the enzymes *in vivo* and does not directly inhibit enzyme activity (data not shown).

Inhibition of caspase activation by H_2O_2 was confirmed by Western blot analysis of cell extracts taken from cells treated for various times with VP-16 \pm H_2O_2 . These immunoassays look for proteolytic cleavage of known intracellular caspase substrates (22). The data in Fig. 7 show the results obtained using antibodies specific for 7 different caspase substrates: caspases-3 and -2 themselves (CPP-32 and ICH-1, respectively), a 470-kDa DNA-dependent protein kinase (DNA-PK), a 240-kDa nuclear matrix protein (NuMA), PARP, GDP dissociation inhibitor (D₄-GDI), and lamin B. H_2O_2 alone did not induce cleavage of any of these proteins, consistent with the conclusion that it kills cells through a non-apoptotic mechanism. VP-16 induced cleavage of all of the proteins. H_2O_2 inhibited VP-16-induced proteolysis by 40–90% for each of the proteins examined (determined by densitometric scanning of the blots). Note that cleavage of CPP-32 is only detected as loss of the pro-enzyme band; the cleavage product is rapidly degraded in the cells and thus is not seen by Western analysis. Similar findings have been reported by other researchers (27, 28).

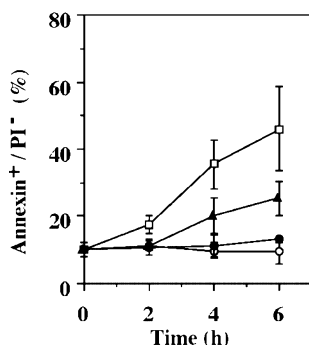


FIG. 5. **Annexin V binding to ST-486 cells treated with H₂O₂, VP-16, or H₂O₂/VP-16.** Cells (5×10^5 /ml) were incubated for 2, 4, and 6 h in the absence (control, ○) or presence of VP-16 (30 μ M, □), H₂O₂ (75 μ M, ●), or H₂O₂ + VP-16 (▲). The cells were incubated with FITC-labeled annexin V and PI and analyzed by FACS as described under "Materials and Methods." The graph shows the proportion of cells that are annexin V-positive and PI-negative (mean \pm S.D. of three separate experiments).

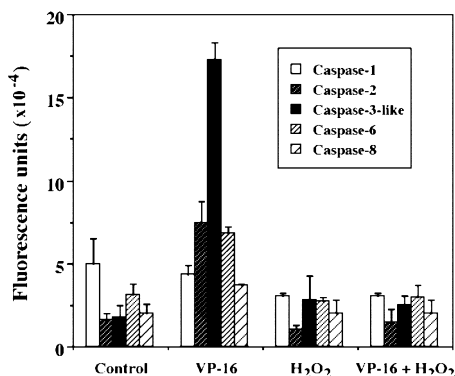


FIG. 6. **Caspase activities in extracts from cells treated with VP-16 in the absence or presence of H₂O₂.** JLP 119 cells (5×10^5 /ml) were treated with VP-16 (5 μ M), H₂O₂ (75 μ M), or both for 4 h. Enzyme activities were measured using fluorogenic sequence-specific substrates for each caspase as described under "Materials and Methods." "Caspase-3-like" refers to all DEVDase activities. Control values are from untreated cells. The results represent the mean \pm S.D. of two experiments carried out in triplicate.

H₂O₂ Inhibits Apoptosis by Lowering Intracellular ATP Levels—Treatment of cells with H₂O₂ is known to cause a rapid depletion of NAD and ATP levels, in part through activation of poly(ADP)ribosylation (6, 10). The fact that H₂O₂ inhibited so many steps in apoptosis and that the inhibition could be overcome by PARP inhibitors suggested that H₂O₂ may be acting by lowering cellular energy stores. To test this hypothesis, we examined the effects of H₂O₂ on cellular ATP levels in JLP 119 cells using a luciferin-luciferase-based chemiluminescence assay (20). The results are shown in Fig. 8. At the subtoxic concentration of 25 μ M (Fig. 4), H₂O₂ had no effect on ATP levels in the cells. At 50 μ M, which induces both pyknosis/necrosis and apoptosis, there is a transient drop in ATP levels, which returns to control levels in 4 h. Concentrations of 75 and 100 μ M, which kill cells entirely by pyknosis/necrosis and completely inhibit VP-16-induced apoptosis cause a rapid drop (within 30 min) in ATP, which remains low throughout the duration of the incubation. No attempt was made to measure ATP levels after an overnight incubation because the cells lose their permeability barrier, thus precluding accurate assessment of intracellular ATP levels.

In order to determine whether the effect of H₂O₂ on ATP levels is responsible for its ability to inhibit apoptosis, conditions were developed so that we could either 1) prevent the drop in ATP and see if H₂O₂ is still able to inhibit apoptosis or 2)

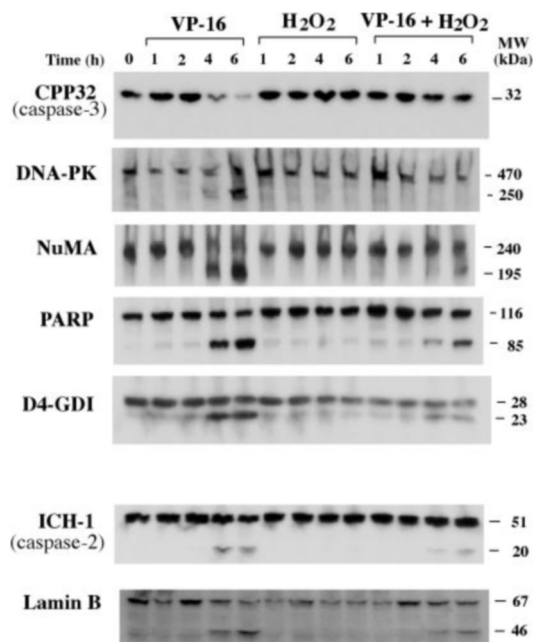


FIG. 7. **Western blot analyses of caspases-2 and -3 and their cellular substrates.** JLP 119 cells (5×10^5 /ml) were treated with VP-16 (5 μ M), H₂O₂ (75 μ M), or both VP-16 and H₂O₂ for 1, 2, 4, and 6 h. Total cell lysates (10^6 cells/lane) were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot immunoassay using antibodies specific for the proteins indicated in the figure. The molecular weights of intact and cleaved proteins are indicated on the right.

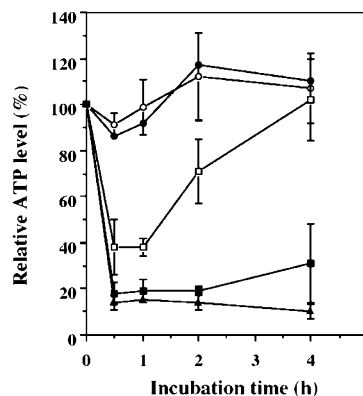


FIG. 8. **ATP levels in JLP 119 cells after exposure to H₂O₂.** JLP 119 cells (5×10^5 /ml) were exposed to different concentrations of H₂O₂ for the times indicated and then assayed for ATP content. Results are expressed as percentage of control values, which averaged 0.85 ± 0.02 nmol of ATP/ 10^6 cells (mean \pm S.D. for three experiments). ○, control; ●, H₂O₂ (25 μ M); □, H₂O₂ (50 μ M); ■, H₂O₂ (75 μ M); ▲, H₂O₂ (100 μ M).

recreate the drop in ATP with a different agent to see if this inhibits apoptosis. As shown in Fig. 9, the drop in cellular ATP induced by 75 μ M H₂O₂ could be completely inhibited by co-treating the cells with the PARP inhibitor 4HQ. Similar results were obtained with 3AB (data not shown). The effect of H₂O₂ on cellular ATP levels could be mimicked by treating the cells with the ATP synthesis inhibitor oligomycin in glucose-free medium (to prevent ATP synthesis through glycolysis) (Fig. 9).

Using these manipulations to control cellular ATP levels, we found that depletion of ATP with oligomycin mimicked the effects of H₂O₂ while protection of cellular ATP by co-treatment with 4HQ overcame the effects of H₂O₂, thus suggesting that H₂O₂ inhibits apoptosis by reducing cellular ATP levels. This was demonstrated at two time points (4 and 22 h) following H₂O₂ treatment (Fig. 10). When cells were treated for 4 h with 5 μ M VP-16 in the presence of 75 μ M H₂O₂, apoptosis was

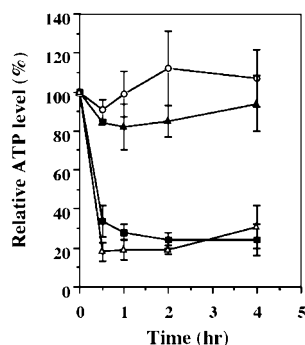


FIG. 9. **Effects of oligomycin and 4HQ on cellular ATP levels.** JLP 119 cells ($5 \times 10^5/\text{ml}$) were incubated with H_2O_2 ($75 \mu\text{M}$) \pm 4HQ ($50 \mu\text{M}$) in glucose-containing medium, or with oligomycin ($10 \mu\text{M}$) in glucose-free medium. Intracellular ATP concentrations were measured at the times indicated and expressed as percentage of untreated controls. \circ , control; \blacktriangle , H_2O_2 + 4HQ; \blacksquare , oligomycin; \triangle , H_2O_2 . Data are means \pm S.D. of triplicate determinations.

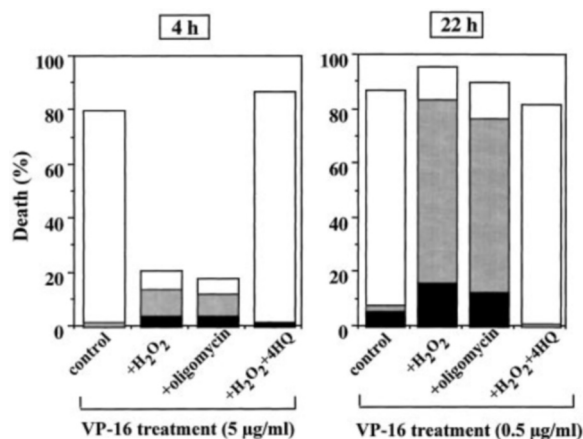


FIG. 10. **Effects of oligomycin and 4HQ on cell killing by VP-16 and H_2O_2 .** JLP 119 cells ($5 \times 10^5/\text{ml}$) were challenged with VP-16 ($5 \mu\text{g}/\text{ml}$ for 4 h or $0.5 \mu\text{g}/\text{ml}$ for 22 h), under ATP-depleting conditions ($75 \mu\text{M}$ H_2O_2 or $10 \mu\text{M}$ oligomycin), or under high ATP conditions (control or co-treatment with $75 \mu\text{M}$ H_2O_2 and $50 \mu\text{M}$ 4HQ). \square , apoptotic; \blacksquare , pyknotic/necrotic; \blacksquare , necrotic. Cell death was quantified by fluorescence microscopy as described under "Materials and Methods."

profoundly inhibited. Note that since H_2O_2 -treated cells do not appear dead (PI-positive) under the fluorescence microscope until much later time points (12–22 h), the level of cell death observed at this time point was also dramatically decreased. The same effect was achieved by depleting cellular ATP with oligomycin. The ability of VP-16 to induce apoptosis in the presence of H_2O_2 was restored by preventing the drop in ATP with 4HQ. Similar results were obtained when cells were examined after an overnight incubation with $0.5 \mu\text{g}/\text{ml}$ VP-16 except that the overall level of cell death was higher: in the presence of H_2O_2 or oligomycin, the mode of cell death was converted from apoptosis to pyknosis/necrosis and this effect could be overcome by preventing the drop in ATP with 4HQ.

DISCUSSION

The results show that H_2O_2 inhibits the ability of agents such as the chemotherapy drug VP-16 or the calcium ionophore A23187 to induce apoptosis in Burkitt's lymphoma cells. The effect of H_2O_2 on cell death is dominant. Thus, cells treated with VP-16 or A23187 in the presence of H_2O_2 resemble cells treated with H_2O_2 alone. Cells killed in the presence of 75 – $100 \mu\text{M}$ H_2O_2 are primarily pyknotic/necrotic. They show none of the classical markers of apoptosis but also are not typically necrotic since the nuclei are somewhat condensed instead of swollen. The biochemical steps that lead to this nuclear mor-

phology are not known but should be examined since they may be common to both apoptosis and necrosis (29–31). The main physiological significance of the effect of oxidative stress on the apoptotic machinery will be found in whether dead cells killed in the presence of H_2O_2 induce an inflammatory response, *i.e.* whether they are removed by phagocytosis without affecting the surrounding tissues like apoptotic cells (3, 4) or whether they leak their cell contents into the extracellular space and induce an inflammatory response like necrotic cells (3).

In investigating the mechanism whereby H_2O_2 modulates cell death, we found that H_2O_2 has such a global effect in inhibiting apoptosis because it acts by depleting cellular energy stores rather than by inhibiting a unique enzyme or factor in the apoptotic machinery. Apoptosis is known to be an active process requiring intracellular ATP (3, 32–34). In the absence of sufficient ATP, cells treated with toxic levels of various agents will die by necrosis instead of apoptosis (31, 35). In these studies, artificial means were employed to manipulate cellular ATP levels (*e.g.* oligomycin treatment). Our results provide evidence that normal metabolic factors, oxidants, that are generated under a wide range of circumstances *in vivo* (36) can determine whether or not cells die by apoptosis. H_2O_2 is a pivotal oxidant since it is generated from nearly all sources of oxidative stress. Due to its structural similarity to water and low innate reactivity, H_2O_2 can diffuse freely in and out of cells and through tissues. Tissue concentrations of H_2O_2 during inflammation have been estimated to reach millimolar levels. Thus, it is relevant that this ubiquitous oxidant can have such a potent effect on progression of apoptosis.

The ability of inhibitors of poly(ADP-ribosylation) to protect ATP levels suggests that H_2O_2 causes the drop in ATP primarily by activating PARP. This occurs as a result of the single-strand DNA breaks induced so potently by H_2O_2 (6, 7, 10). The depletion of NAD^+ that ensues prevents activity of glyceraldehyde-3-phosphate dehydrogenase (11). The result is inhibition of glycolysis at a stage where ATP has been consumed but not yet resynthesized (either through the downstream steps of glycolysis or through mitochondrial oxidative phosphorylation). The overall effect is a precipitous drop in ATP levels. H_2O_2 can also inhibit ATP synthesis by other mechanisms, including direct oxidative inactivation of mitochondrial ATP synthase (37) and possibly glyceraldehyde-3-phosphate dehydrogenase (38). This may or may not play a role in our experimental system.

Loss of ATP probably contributes to the induction of cell death by H_2O_2 but it is not only mechanism whereby H_2O_2 kills the cells, since protection of intracellular ATP concentrations with 3AB and 4HQ still results in cell death; the cells just die by apoptosis instead of pyknosis/necrosis. This finding is consistent with earlier reports showing that H_2O_2 can kill cells by mechanisms that do not depend upon ATP depletion or activation of PARP (39, 40). Additional pathways responsible for H_2O_2 -induced cell death have been defined (41) and probably depend on iron-mediated formation of secondary radicals (42, 43). Further research will be required to determine where these pathways function in the cell death machinery.

Previous researchers have reported that H_2O_2 inhibits caspase activity in Jurkat cells (44) and concluded that H_2O_2 might act by inhibiting the caspases directly. Our data do not support such a conclusion. None of the caspases was inhibited when H_2O_2 was added directly to caspase-containing cell extracts. In addition, we found that Jurkat cells behaved similarly to Burkitt's lymphoma cells in that Fas-mediated apoptosis could be inhibited by addition of H_2O_2 , and this was accompanied by a dramatic drop in ATP levels. Prevention of the drop in ATP in the Jurkat cells with 3AB allowed apoptosis

to occur even in the presence of H_2O_2 .² Thus, a mechanism requiring direct inhibition of caspase activity need not be invoked to explain how H_2O_2 inhibits apoptosis. The ATP level appears to be the pivotal determinant for whether apoptosis can proceed.

There are numerous reports in the literature showing that oxidants kill cells by inducing apoptosis (12, 15, 45–48), and induction of oxidative stress is often proposed as a common mechanism whereby diverse agents induce apoptosis (13, 17, 49). Our results (this report and Ref. 14) and those of others (50–52) challenge the generalizability of this view. Many of the early papers suggesting that oxidants kill cells by inducing apoptosis were not quantitative (15, 45, 46), *i.e.* apoptotic cells were found (by microscopy or detection of DNA ladders) among the population of cells treated with H_2O_2 but the percentage of cells dying by apoptosis *versus* necrosis was not determined. Thus, although it is clear that oxidants such as H_2O_2 can induce apoptosis, this may not be the primary mode of cell death and may occur only under a narrow set of circumstances. In our studies, we found that apoptosis was only induced to a significant degree at a very narrow concentration range around 50 μM H_2O_2 . Our data suggest that the reason for this resides in the extent to which H_2O_2 depletes cellular ATP levels; at 50 μM H_2O_2 , the drop in ATP is transient, thus allowing a portion of the cells to die by apoptosis. At higher H_2O_2 concentrations (75–100 μM), the drop in ATP appears to be irreversible, thus leading to pyknotic/necrotic cell death. The concept that low concentrations of a toxin induce apoptosis while high concentrations induce necrosis is not novel (12, 15). Our results provide an explanation for these findings, particularly as they apply to mechanisms of oxidant-induced cell death. In considering the application of these results to other cell systems, it is important to remember that different cell types have different susceptibilities to H_2O_2 toxicity. Thus, the absolute concentrations of H_2O_2 required to inhibit apoptosis will vary from cell to cell. As H_2O_2 is likely to lower ATP levels in most cells, it is also likely to inhibit apoptosis in most cells.

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