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Inhibition by Ascorbic Acid of Apoptosis Induced by Oxidative Stress in HL-60 Myeloid Leukemia Cells

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ABSTRACT. The human myeloid leukemia cell line HL-60 transports the oxidized form of ascorbic acid, dehydroascorbic acid (DHA), and accumulates reduced ascorbic acid. We studied the effect of ascorbic acid loading on apoptosis induced by serum- and glucose-free culture and by oxidative stress induced by H_2O_2. Uptake accumulation studies indicated that incubation of HL-60 cells with DHA resulted in the accumulation of intracellular ascorbic acid which decreased with time when cells were incubated in DHA-free medium. Exposure of HL-60 cells to increasing concentrations of H_2O_2 resulted in dose-dependent intracellular accumulation of peroxides, as determined by the use of the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), which was accompanied by a decrease in intracellular ascorbic acid and an increase in apoptosis. A dramatic decrease in intracellular ascorbic acid was noted when preloaded HL-60 cells were exposed to 150 μM H_2O_2 (the concentration dropped from 5.2 ± 0.6 mM to 3.6 ± 0.1 mM in cells preincubated with 150 μM DHA). A dose-dependent protective effect of DHA was observed. Ascorbic acid loading also provided strong protection from apoptosis associated with serum- and glucose-free culture. Flow cytometry studies showed that exposure of HL-60 cells to 150 μM H_2O_2 resulted in decreased Bcl-2 expression that was associated with enhanced apoptosis (up to 33.6 ± 2.6%). No significant variation of Bcl-2 expression was measured following exposure of HL-60 cells, loaded with ascorbic acid, to 150 μM H_2O_2 and only a slight increase (up to 10.1 ± 3.1%) in apoptosis. These findings indicate that ascorbic acid can inhibit apoptosis induced by oxidative stress in HL-60 cells. BIOCHEM PHARMACOL **57**;7:823–832, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. apoptosis; oxidative stress; ascorbic acid

Humans cannot synthesize ascorbic acid (vitamin C) [1], so it must be provided exogenously in the diet and transported intracellularly. Until recently, it was not known how ascorbic acid is transported into human tissues [2]. Recent studies have shown that ascorbate and DHA‖ are transported into human neutrophils by two distinct mechanisms [3]. Other studies performed with the HL-60 cell line that served as a model for human myeloid cells have shown that these cells transport ascorbic acid only in the form of DHA [4, 5]. Once inside the cell, DHA is reduced to ascorbic acid, a non-transportable moiety. Ascorbic acid is known to accumulate in tissues [2, 3] and is crucial to health [6–8]; however, it is not clear how the vitamin functions intracellularly in most cell types. For example, ascorbic acid is necessary for normal leukocyte function, but the precise role it plays in leukocyte biology is uncertain.

The antioxidant properties of ascorbic acid have been emphasized previously [9, 10]. Ascorbic acid, however, can act as an antioxidant or a pro-oxidant, for example, in the Fenton reaction with iron [11]. We performed a study of the fate of the intracellular accumulated ascorbic acid in HL-60 cells and addressed the role of ascorbic acid as an antioxidant in these cells by testing the hypothesis that ascorbic acid can ameliorate apoptosis induced by oxidative stress. We treated HL-60 cells with hydrogen peroxide, inducing a reproducible incidence of apoptotic death. Ascorbic acid loading of these cells ameliorated apoptosis induced by low concentrations of hydrogen peroxide, suggesting that ascorbic acid can protect cells from oxidative stress by neutralizing the toxic action of pro-oxidants.

The proto-oncogene Bcl-2 was found to have the ability to block apoptosis induced by a wide variety of stimuli. The mechanism by which the Bcl-2 protein extends cell survival is unclear. Although in recent studies evidence was pro-

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‖ *Abbreviations:* DCFH-DA, 2′7′-dichlorofluorescind-diacetate; DHA, dehydroascorbic acid; DCF, 2′7′-dichlorofluorescein; DTT, dithiothreitol; FBS, fetal bovine serum; IMDM, iscove's modified Dulbecco's medium; TUNEL, Tdt-mediated dUTP-biotin nick end labeling; and RT, room temperature.

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duced against the hypothesis that Bcl-2 inhibits apoptosis through antioxidant effects [12–14], other studies have suggested that Bcl-2 acts in an antioxidant pathway, suppressing oxygen radical-induced lipid peroxidation [15] or decreasing the generation of reactive oxygen species [16]. Exposure of cells to low doses of H_2O_2 resulted in increased protein synthesis in HA-1 cells [17] and in upregulated expression of Bcl-2 in myeloma cells [18], in primary human fibroblasts [19], and in OCI/AML-2 cells [20]. Therefore, we wondered whether exposure of ascorbic acid-loaded and control HL-60 cells to low doses of H_2O_2 would affect Bcl-2 synthesis.

Our findings show that exposure of control cells to low concentrations of H_2O_2 enhanced Bcl-2 expression, while no such enhanced expression was observed in ascorbic acid-loaded cells. Exposure of control cells to higher H_2O_2 concentrations resulted in decreased Bcl-2 expression and enhanced apoptosis, while no decreased Bcl-2 expression nor enhanced apoptosis was observed in ascorbic acid-loaded cells exposed to the same concentration of hydrogen peroxide.

MATERIALS AND METHODS

Cell Culture and Uptake Studies of DHA

HL-60 cell cultures were maintained in IMDM (GIBCO) supplemented with 10% FBS (HyClone Laboratories [21]). Cells were resuspended in incubation buffer (15 mM Hepes pH 7.6, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.05 mM DTT) at a final concentration of 2×10^6 cells/mL. Cell viability was greater than 95% as determined by trypan blue exclusion.

Ascorbic acid (Sigma Chemical Co.) was dissolved in phosphate buffer (KH_2PO_4 0.1 M, Na_2HPO_4 4 mM, EDTA 0.5 mM) pH 5.6. Various concentrations of ascorbic acid were incubated with 16 to 60 U ascorbate oxidase at room temperature for 2 min [4]. For uptake studies, 0.5 mL of incubation buffer containing 2×10^6 cells/mL was added to 0.5 mL of incubation buffer containing the oxidized ascorbic acid (final concentration 25–250 μM DHA). Cells were incubated for 5–90 min. Solutions of ascorbic acid were prepared daily. For ascorbic acid uptake assays, we used a mixture of unlabeled ascorbic acid and 0.5 μCi of L-[^{14}C]-ascorbic acid (specific activity 6.6 mCi/mmol, NEN-DuPont) which was oxidized by ascorbate oxidase. The final concentration of DHA was 150 μM. The cells were incubated for 5–60 min at 37° and then collected by centrifugation and washed twice by centrifugation in cold (4°) stopping solution ([PBS] without Ca^{++} and Mg^{++}). Cells were then solubilized in 0.2 mL of 50 mM Tris–HCl pH 7.8 containing 1% SDS. The cell-associated radioactivity was determined by scintillation spectrometry. A sample in which the cells were immediately centrifuged in the presence of cold stopping solution was used as a control for non-specifically trapped radioactivity.

Measurement of Intracellular Ascorbic Acid

Cells were washed twice with PBS at 4°, and centrifuged. One hundred μL of 0.1 M perchloric acid was added to each pellet (2×10^6 cells/sample). The cells were then homogenized by sonication, centrifuged for 10 min at 16,600*g* in the cold (4°) as previously described [22] and stored at −70° until analyzed by HPLC. HPLC analysis was performed according to the following procedure. Briefly, the samples were thawed on ice and centrifuged for 10 min at 16,600*g* in the cold. A 20 μL aliquot of the supernatant was injected into the HPLC system. Separation and detection were carried out on a reverse-phase paired ion HPLC with a Supelcosil LC-308 (5 micron) column (Supelco) and Waters 996 Photodiode Array Detector set at the 265 nm. The data was recorded and processed using the Millennium 2000 program (Waters). The mobile phase consisted of 20 mM sodium acetate buffer, 200 mg/L EDTA, and 40 mM *n*-octylamine. The final pH of the mobile phase was 3.6 and the flow rate was 1.0 mL/min. Ascorbic acid standards consisted of 0.5 mg/L and 1.0 mg/L ascorbic acid in 0.1 M perchloric acid.

Treatment of Cells with Hydrogen Peroxide

Exponentially growing HL-60 cells were resuspended in incubation buffer at a concentration of 1×10^6 cells/mL and 25–250 μM DHA was added as described above. The cells were incubated at 37° with 5% CO_2 for 15 min. Control cells were incubated with 0.05 mM DTT. Following incubation, the cells were washed, resuspended in IMDM supplemented with 3% BSA (Boehringer Mannheim) 1 μM insulin, and exposed to 50–300 μM H_2O_2. The cells were incubated for 4–14 hr at 37° with 5% CO_2, and evaluated for apoptosis.

In other experiments, cells were incubated with 50 μM or 150 μM DHA for 15 min, washed, resuspended in IMDM, supplemented with 3% BSA, 1 μM insulin as described above, and exposed to 25–150 μM H_2O_2. The cells were incubated for 1–8 hr at 37° with 5% CO_2, and the intracellular concentration of ascorbic acid was analyzed by HPLC.

Apoptosis Studies

Conventional cytocentrifuge preparations were made, cells were fixed, stained with Giemsa and analyzed by light microscopy. Apoptotic cells were easily identifiable on Giemsa-stained cytocentrifuge samples by chromatin condensation, which resulted in an intensely basophilic nucleus and nuclear fragmentation [23]. At least 200 cells from randomly selected fields were counted. Additional analysis of apoptosis was performed by Nomarsky differential interference contrast microscopy and by the method of TUNEL [24]. In brief, centrifuge preparations were made, and the cells were fixed in 4% buffered formaldehyde and washed with PBS, immersed in TDT buffer (30 mM Trizma

base pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TDT (0.3 e.u./μL) and biotinylated dUTP in TDT buffer were then added to cover the cells and then incubated in humid atmosphere at 37° for 60 min. The reaction was terminated by transferring the slides to 2XSSC (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at RT. The cells were washed with PBS, covered with 2% solution of BSA in PBS for 15 min at RT. The cells were covered with fluorescein isothiocyanate streptavidin (Vector Lab.) diluted 1:100 in PBS, incubated for 30 min at 37°, immersed for 5 min in PBS, and stained with 4′,6-diamidino-2-phenylindole (Sigma Chemical Co.) for about 30 min at 37°.

Isolation and Analysis of DNA

Cells were incubated in 150 mM NaCl, 25 mM EDTA containing 100 μg/mL proteinase K, and 0.2% SDS at 50° for three hr, extracted twice with phenol chloroform and once with chloroform [25]. DNA was precipitated with 66% ethanol in the presence of 0.55 M CH_3COONH_4, rinsed with 70% ethanol and dried. The DNA was dissolved in 10 mM Tris–HCl (pH 8.0) containing 1 mM EDTA, then treated with 20 μg/mL RNAase for 30 min at 37°. Approximately 1–10 μg of DNA was placed on 1.8% (wt/vol) agarose gel containing 0.5 μg/mL ethidium bromide. Electrophoresis was carried out for 5 hr at 90 V, after which the gel was photographed under ultraviolet light.

Fluorescent Measurement of Intracellular Peroxides

HL-60 cells (5×10^6/mL) were loaded with 5 μM DCFH-DA (Molecular Probes) (dissolved in DMSO at 2000 ×) for 15 min with horizontal agitation in a shaking water bath at 37° [15, 26]. After a 15-min preincubation, the cells were washed by centrifugation and dispersed in PBS (1×10^6 cells/mL). Hydrogen peroxide (25–150 μM) was added to the cells and incubation continued for 60 min. The cells were then washed by centrifugation, brought to a concentration of 5×10^6/mL and sonicated using 3 × 12-sec sonications with an Artek sonifier (Artek System Corporation). The sonicates were centrifuged at 27,000*g* for 10 min at 4°, and the fluorescent supernatant was used for spectral analysis of the oxidized dye product [26]. Excitation and emission spectra were determined for reagent DCF (Polysciences, Inc.). For the reagent DCF spectrum, a 1 mM DMSO stock solution of the dye was diluted in PBS to a final concentration of 500 nM and placed in a 3-mL cuvette with a magnetic stirring flea. The excitation and emission spectra were then determined on an SLM Amnico (SLM Instruments, Inc.) spectrofluorometer. Serial dilutions of reagent DCF were monitored on the spectrofluorometer to obtain a standard curve of fluorescence per nmol DCF. The mean DCF concentration in the supernatant of the sonicated HL-60 cells could be ascertained from the standard curve.

Flow Cytometry for BCL-2 Expression

HL-60 cells were preincubated in incubation buffer in the presence or absence of 150 μM DHA for 15 min. The cells were washed, suspended in culture medium, exposed to 50–150 μM H_2O_2 for 6-hr, washed and cultured for 24 hr. Fluorescence-activated cell sorter (FACS) analysis for Bcl-2 expression was performed as previously described [18] with slight modifications. Briefly, PBS-washed cells were treated with 0.25% paraformaldehyde for 15 min at RT, washed and resuspended in 70% methanol for 1 hr at 4°. After washing ×2, cells were incubated with anti-human Bcl-2 monoclonal antibody (DAKO) as previously described [18]. They were then exposed to the secondary antibody fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat antimouse anti-mouse immunoglobulin G (Sigma Chemical Co.). Cells were analyzed by flow cytometry with a FACScan apparatus (Beckton Dickinson). The corresponding control consisted of identical staining except for the use of an irrelevant mouse immunoglobulin G of identical isotype (Dako) instead of the anti-Bcl-2 antibody. Intensity of fluorescence was expressed as mean channel fluorescence. Statistical significance was determined by using the Student's *t*-test.

RESULTS

Uptake of DHA and Intracellular Accumulation of Ascorbic Acid

We measured the uptake of DHA (^{14}C-labeled substrate) (150 μM) by the HL-60 cells. Ascorbic acid was quantitatively converted to DHA with the addition of ascorbic acid oxidase to the incubation buffer immediately prior to the uptake assay. As can be seen in Fig. 1A, radioactivity rapidly accumulated in the cells, reaching a maximum at 30 min. We then performed quantitative HPLC analysis of ascorbic acid which has accumulated in the cells. An estimated intracellular volume of 0.3 μL per 10^6 HL-60 cells was used to express the concentration of ascorbic acid in the cells [4]. Fig. 1B shows that cellular accumulation of ascorbic acid occurred with time and reached a plateau at 30 min. At the end of the 30-min incubation period in the presence of 150 μM DHA, the HL-60 cells contained 10.6 ± 0.7 mM ascorbic acid.

Ascorbic Acid Loading Protection from Apoptosis Induced by Culture in Serum- and Glucose-free Medium

Since previous studies have shown that HL-60 cells transport DHA through glucose transporters [4, 5] and that glucose inhibits ascorbic acid transport in human neutrophils [27], the uptake accumulation experiments described above were performed in incubation buffer not containing glucose. We examined the effect of prolonged incubation of cells in this incubation buffer on apoptosis. Since nuclear fragmentation appears 2 hr after cells are exposed to oxidative stress and complete cellular breakup into apopto-

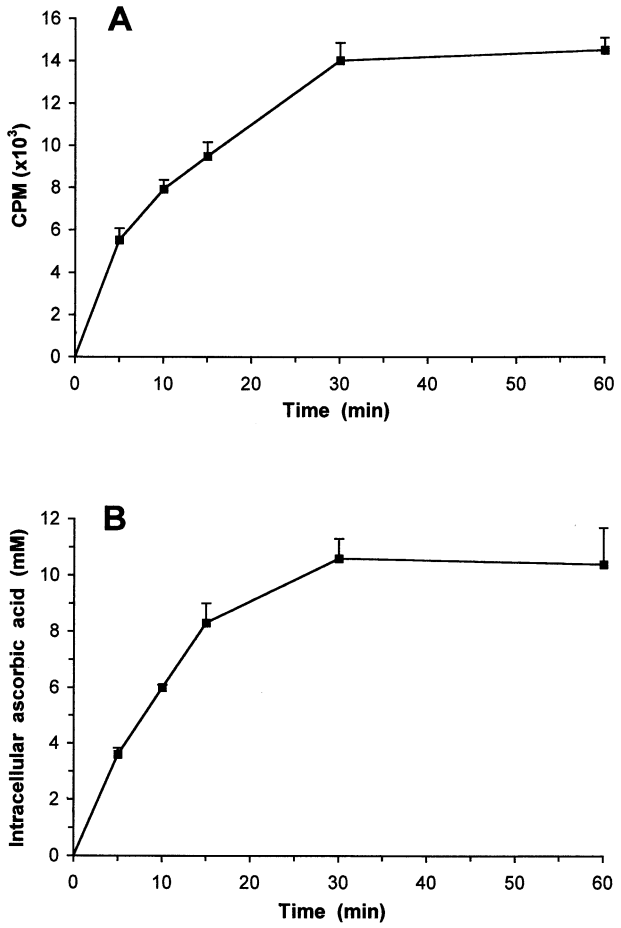


FIG. 1. Transport of DHA, accumulation of ascorbic acid, and apoptosis. (A) Uptake of DHA. HL-60 cells were incubated for various periods of time in incubation buffer containing 75 μM radioactive DHA, 75 μM unlabeled DHA, and 0.05 mM DTT. The cell-associated radioactivity was determined by scintillation spectrometry. Results correspond to the mean ± SE of three samples. Two additional experiments showed similar results. (B) Intracellular accumulation of ascorbic acid. HL-60 cells were incubated for various periods of time with 150 μM DHA, 0.05 mM DTT, and the intracellular accumulation of ascorbic acid was analyzed by HPLC. Using an internal volume of 0.3 μL per 10^6 cells, the data were expressed as intracellular concentrations of ascorbic acid.

tic bodies occurs within a 4-hr time-span [28], HL-60 cells were preincubated in incubation buffer for 15–90 min, after which time they were resuspended in IMDM containing 10% FBS and incubated at 37° for 4 hr before assessing apoptosis. The percentage of apoptotic cells was monitored on Giemsa-stained cytocentrifuge preparations. Cells grown in IMDM containing 10% FBS contain 3 ± 0.3% apoptotic cells. Figure 2 shows that preincubation of HL-60 cells in incubation buffer for 15 min did not affect the percentage of apoptotic cells, while an increase in the percentage of apoptotic cells occurred following prolongation of the preincubation period. Thus, preincubation of the cells for 90 min resulted in 15.2 ± 1.8% of apoptotic cells. As preincubation of the cells in incubation buffer containing 150 μM DHA for 60 or 90 min reduced apoptosis 3.2- and 2.6-fold, respectively ($P < 0.05$), preincubation in the presence of DHA had a protective effect. The continuation of the experiments was performed following incubation of the cells with DHA for 15 min.

Role of Ascorbic Acid Loading in Resistance to Apoptosis Induced by H_2O_2

We analyzed the protective effect of ascorbic acid loading on apoptosis induced by various concentrations of H_2O_2. In addressing this issue, HL-60 cells were preincubated in incubation buffer containing various concentrations of DHA, and the cells were exposed to increasing concentrations of H_2O_2 for up to 14 hr. The percentage of apoptotic cells was monitored on Giemsa-stained cytocentrifuge preparations. Figure 3A indicates that treatment of cells that were preincubated with 150–300 μM H_2O_2 for 4 hr (i.e. in incubation buffer not containing DHA) resulted in a dose-dependent increase in the percentage of apoptotic cells (15–44%). However, HL-60 cells preincubated with 50 μM DHA and exposed to 150 or 200 μM H_2O_2 for 4 hr showed a significant diminished apoptotic response (5% and 16%, respectively, as compared to 15% and 27%, respectively, in cells preincubated in buffer not containing DHA) ($P < 0.05$) (Fig. 3A). At higher H_2O_2 concentrations, preincubation with 50 μM DHA did not inhibit H_2O_2-induced apoptosis in HL-60 cells.

A dose-dependent protective effect of DHA was observed following preincubation of HL-60 cells for 15 min with increasing concentrations of DHA and cell exposure to 200 μM H_2O_2 for 4 hr. Figure 3B shows that preincubation of cells with 25–250 μM DHA significantly pro-

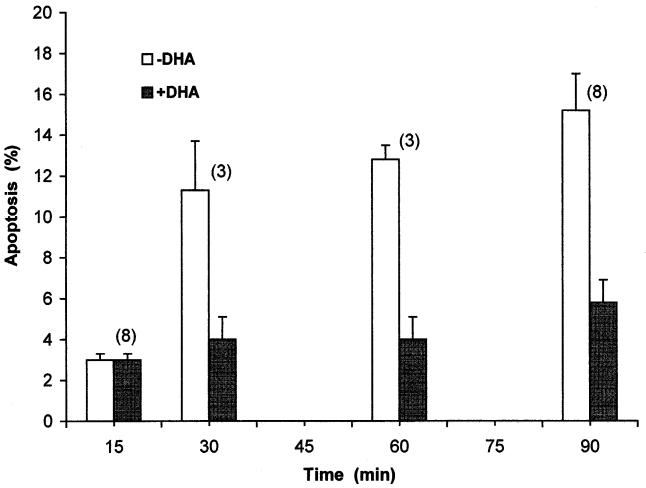
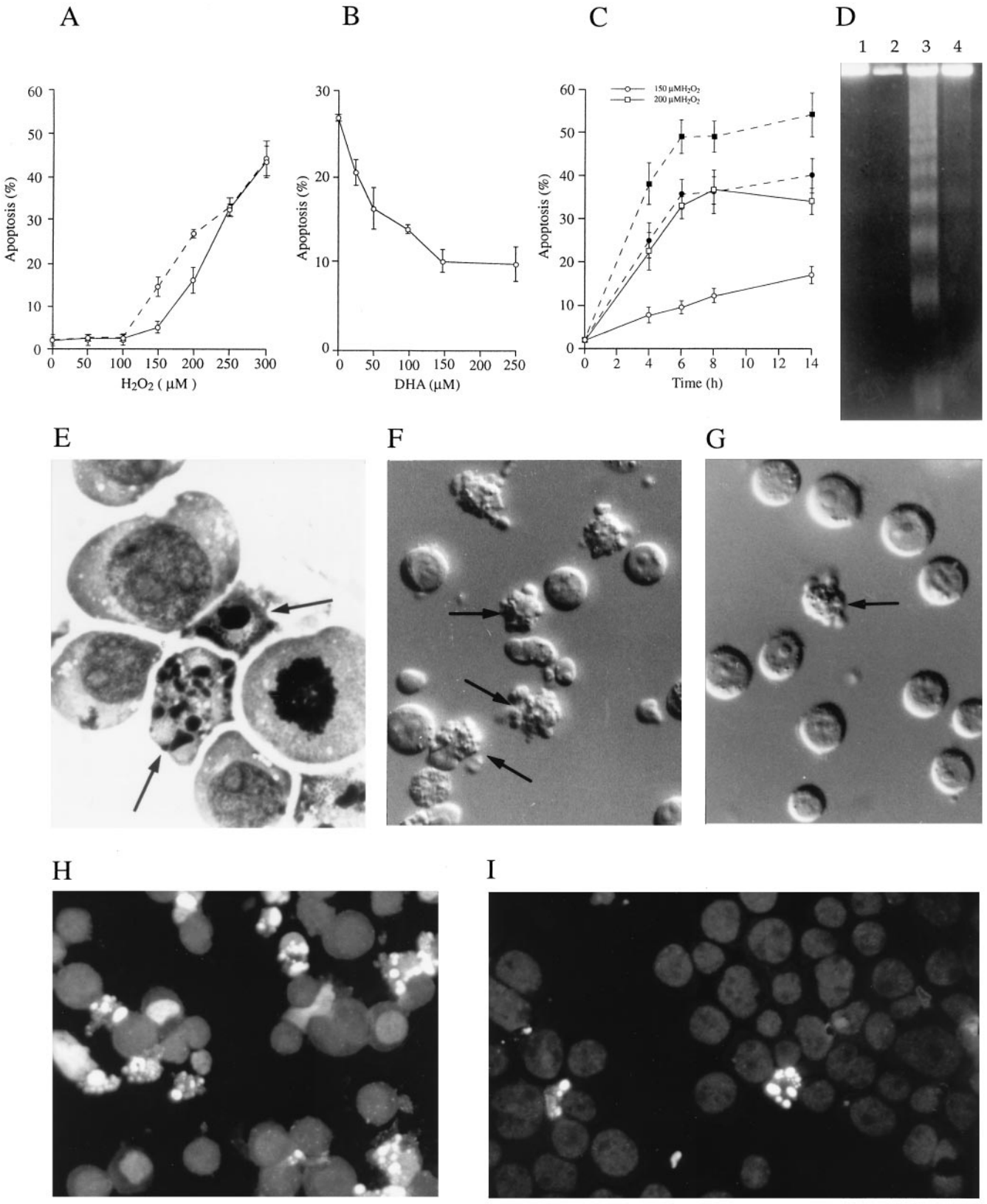


FIG. 2. Apoptosis induced in HL-60 cells cultured in incubation buffer (not containing glucose or FBS). Cells were prepared by culture with incubation buffer for the indicated times in the absence or presence of 150 μM DHA and transferred to IMDM containing 10% FBS, and incubated for 4 hr. The percentage apoptosis in cells was determined using morphological criteria on Giemsa-stained cytocentrifuge preparations. Numbers in parentheses indicate the number of experiments performed (in duplicate).


A
B
C
D
1 2 3 4
Apoptosis (%)
H_2O_2 (μM)
DHA (μM)
Time (h)
150 μM H_2O_2
200 μM H_2O_2
E
F
G
H
I

tected apoptosis induced by 200 μM H_2O_2 ($P < 0.05$). No protective effect of DHA could be observed when HL-60 cells were preincubated with 50–250 μM DHA and exposed for 4 hr to 250 or 300 μM H_2O_2 (data not shown). HL-60 cells were preincubated with 150 μM DHA for 15 min, exposed to 150 or 200 μM H_2O_2, and the percentage of apoptotic cells was monitored at various time points. Figure 3C shows that a significant protective effect of DHA was observed when cells were exposed to 150 or 200 μM H_2O_2 for up to 14 hr ($P < 0.05$). In addition to Giemsa-stained cell examination (Fig. 3E), further analysis of cell morphology was performed by Nomarsky differential interference contrast microscopy (Fig. 3F and G) and by TUNEL (Fig. 3H and I), which confirmed the protective effect of DHA. When the DNA of the cells exposed to 150 μM H_2O_2 for 6 hr was analyzed by gel electrophoresis, cells from cultures preincubated with buffer not containing DHA showed the extensive degradation of DNA to oligonucleosomal fragments which is characteristic of apoptosis (Fig. 3D). The protective effect of DHA is seen.

Cellular Accumulation of Peroxides and Ascorbic Acid; Effect of Time and Exposure to H_2O_2

We examined intracellular peroxide levels and ascorbic acid concentrations in HL-60 cells exposed to H_2O_2. Two experimental approaches were used:

1. To directly assess the intracellular peroxide levels in HL-60 cells exposed to H_2O_2, we used an oxidation-sensitive fluorescent probe DCFH-DA. HL-60 cells were incubated with DCFH-DA, which is deacylated to the non-fluorescent compound DCFH and can be oxidized to the fluorescent compound DCF by a variety of peroxides [15, 26], for 15 min. The cells were then washed, resuspended in PBS, increasing concentrations of H_2O_2 were added (25–150 μM), and incubation continued for 60 min. The cells were then washed and sonicated. DCF fluorescence in the supernatants of the sonicates was measured. Figure 4 indicates that a marked increase in fluorescence intensity occurred upon incubation of the cells with increasing concentrations of H_2O_2. Fluorescence intensity of HL-60 cells exposed to 50 or 150 μM H_2O_2 for 60 min reached levels 3.9- and 5.4-fold higher, respectively, than those of control cells (not exposed to H_2O_2).
2. HL-60 cells were preincubated with 150 μM DHA, increasing concentrations of H_2O_2 were added (25–150 μM), and incubation continued for 60 min. The intracellular ascorbic acid concentration was determined by HPLC. Figure 4 indicates that a decrease in intracellular ascorbic acid concentration occurred upon incubation of the cells with increasing concentrations of H_2O_2. Following exposure of the cells to 50 or 150 μM H_2O_2, the intracellular ascorbic acid concentrations dropped to 71.5 ± 0.2% and 51.7 ± 6%, respectively, of ascorbic acid concentrations of cells not exposed to H_2O_2. The determination of intracellular concentrations of perox-

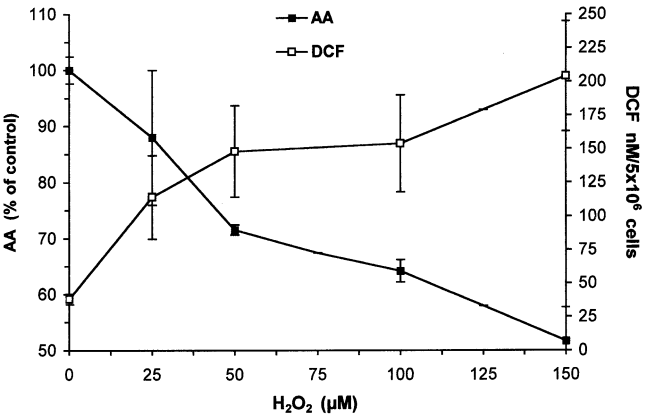


FIG. 4. Oxidation of intracellular DCFH to DCF and accumulation of ascorbic acid in HL-60 cells. HL-60 cells were preincubated with 5 μM DCFH-DA for 15 min, washed, increasing concentrations of H_2O_2 were added, and the cells were incubated for 60 min. Following incubation, the cells were washed and sonicated, and the mean concentration of DCF/5 × 10^6 HL-60 cells was determined on a spectrofluorometer that had been standardized with reagent DCF as described in Materials and Methods. Results correspond to the mean ± SE of 3 experiments. In parallel, HL-60 cells were preincubated with 150 μM DHA, washed, increasing concentrations of H_2O_2 were added, and the cells were incubated for an additional 60 min. Intracellular accumulation of ascorbic acid was analyzed by HPLC. Results correspond to the mean ± SE of three samples.

FIG. 3. Protective effect of DHA against apoptosis induced by H_2O_2. (A) Effect of increasing concentration of H_2O_2 on apoptosis in HL-60 cells. Cells were prepared by culture with incubation buffer in the absence (dotted line) or presence (solid line) of 50 μM DHA for 15 min and transferred to IMDM containing 3% BSA, 1 μM insulin and exposed to 50–300 μM H_2O_2. The percentage apoptosis in these cells was determined using morphological criteria on Giemsa-stained cytocentrifuge preparations following exposure of the cells to H_2O_2 for 4 hr. Data represent mean ± SE of 3 experiments performed in duplicate. (B) Effect of increasing concentrations of DHA. HL-60 cells were preincubated for 15 min with incubation buffer only or 25–250 μM DHA and exposed to 200 μM H_2O_2 for 4 hr. The percentage of apoptosis in cells was determined as described in A. (C) Effect of time. HL-60 cells were preincubated with 150 μM DHA for 15 min (solid line) or incubation buffer only (dotted line) and exposed to 150 or 200 μMH_2O_2 for 4–14 hr. The percentage of apoptosis in cells was determined as described in A. (D) Total DNA from cells preincubated with buffer or 50 μM DHA and exposed to 150 μM H_2O_2 for 6 hr. Lanes 1 and 3 show DNA isolated from cells preincubated with buffer only; Lanes 2 and 4, cells preincubated with 50 μM DHA; Lanes 3 and 4, DNA isolated from cells exposed to 150 μM H_2O_2 for 6 hr; Lanes 1 and 2, control cells. (E) Morphological features of apoptosis induced by 150 μM H_2O_2 for 6 hr (Giemsa-stained cells). Apoptotic cells are indicated with arrows. (F) and (G) Nomarski differential interference contrast microscopy of HL-60 cells preincubated with buffer or 50 μM DHA, respectively and exposed to 150 μM H_2O_2 for 6 hr. (H) and (I) TUNEL staining of HL-60 cells preincubated with buffer or 50 μM DHA, respectively and exposed to 150 μM H_2O_2 for 6 hr.

ides and ascorbic acid was performed in parallel experiments when cell preparations were handled similarly with H_2O_2. We could not determine the concentration of peroxides in HL-60 cells that were preloaded with ascorbic acid, as the latter altered the fluorescence of DCF.

To determine the effect of time on intracellular concentrations of ascorbic acid, cells were preloaded with DHA for 15 min, washed and incubated in DHA-free medium for 4–8 hr. Ascorbic acid concentrations were determined by HPLC. Intracellular ascorbic acid concentrations of control HL-60 cells that were incubated with 50 or 150 μM DHA were 0.74 ± 0.12 mM and 8.6 ± 1.5 mM, respectively (Fig. 5A and B). A dramatic decrease in ascorbic acid concentration occurred with time. Incubation of HL-60 cells preloaded with 50 or 150 μM DHA in medium, not containing DHA, for 8 hr, resulted in a decrease in intracellular ascorbic acid concentration to 35% and 60%, respectively of the ascorbic acid concentration at zero time ($P < 0.05$) (Fig. 5A and B). HL-60 cells were preloaded with 50 or 150 μM DHA for 15 min and exposed to 150 μM H_2O_2 for 4–8 hr. Upon exposure of the cells to H_2O_2 for 4 hr, intracellular ascorbic acid concentration decreased from 0.59 ± 0.04 mM to 0.29 ± 0.02 mM for cells preloaded with 50 μM DHA and from 7.9 ± 1.4 mM to 5.6 ± 0.7 mM ascorbic acid for cells preloaded with 150 μM DHA. Following 8-hr exposure of cells to 150 μM H_2O_2, no intracellular ascorbic acid could be detected in cells preloaded with 50 μM DHA, while the intracellular concentration of ascorbic acid dropped to 3.6 ± 0.1 mM in cells preloaded with 150 μM DHA ($P < 0.05$) (Fig. 5A and B).

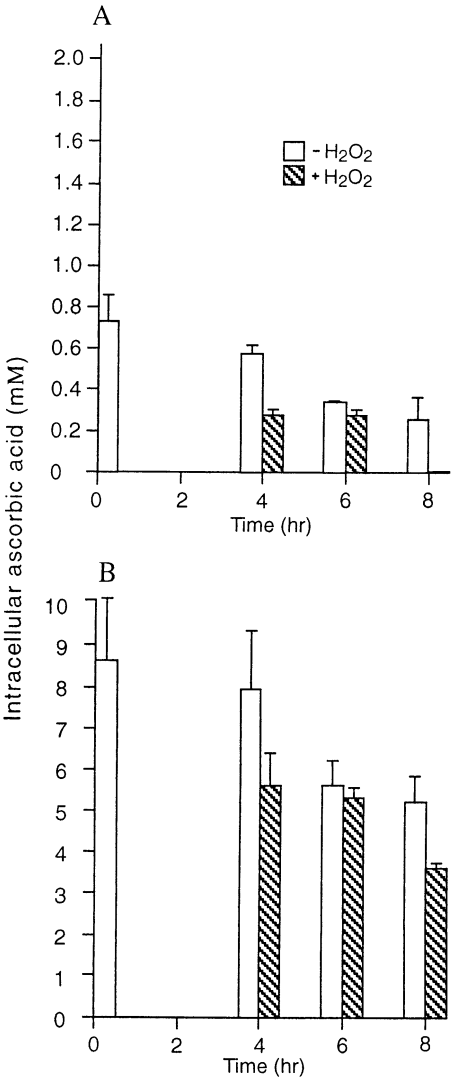


FIG. 5. Effect of time on intracellular concentrations of ascorbic acid. HL-60 cells were incubated for 15 min in incubation medium containing A, 50 μM DHA, or B, 150 μM DHA. Following incubation, the cells were transferred to IMDM containing 3% BSA and 1 μM insulin and incubated for 4–8 hr in the absence or presence of 150 μM H_2O_2. Intracellular accumulation of ascorbic acid was analyzed by HPLC. Using an internal volume of 0.3 μL per 10^6 cells, the data were expressed as intracellular concentrations of ascorbic acid. Results correspond to the mean ± SE of three samples.

Expression of Bcl-2 in Control and Ascorbic Acid-loaded HL-60 Cells Exposed to H_2O_2

Because Bcl-2 plays a central role in protection against apoptosis, Bcl-2 levels were investigated in ascorbic acid-loaded and control cells (not loaded with ascorbic acid). To assess effects of Bcl-2 expression, HL-60 cells were incubated with increasing concentrations of H_2O_2 (50–150 μM) for 6 hr, washed, and recultured for 24 hr, after which cells were analyzed for Bcl-2 content, by flow cytometry, and for apoptosis (on cytospin preparations). As shown in Fig. 6A, exposure of control cells to 50 μM H_2O_2 resulted in a slight upregulated Bcl-2 expression, while preincubation of HL-60 cells with 150 μM DHA for 15 min and their exposure to the same H_2O_2 concentration did not affect Bcl-2 expression. Similar results were obtained following exposure of the control and ascorbic acid-loaded cells to 100 μM H_2O_2 (data not shown). At both H_2O_2 concentrations, there was no effect on the percentage of apoptotic cells. However, exposure of control cells to 150 μM H_2O_2 resulted in decreased Bcl-2 expression (Fig. 6B), and this was associated with enhanced apoptosis up to 33.6 ± 2.6%. Incubation of ascorbic acid-preloaded HL-60 cells with 150 μM H_2O_2 did not affect Bcl-2 expression. Similar Bcl-2 expression was observed following exposure of ascorbic acid-loaded cells to 150 μM H_2O_2 or to medium (Fig. 6B). Exposure of ascorbic acid-preloaded cells to 150 μM H_2O_2 resulted in 10.1 + 3.1% apoptotic cells.

DISCUSSION

Generation of reactive oxygen intermediates is a normal process in aerobic organisms, but at the same time an internal threat to cellular homeostasis can arise from these reactive oxygen intermediates and the by-products gener-

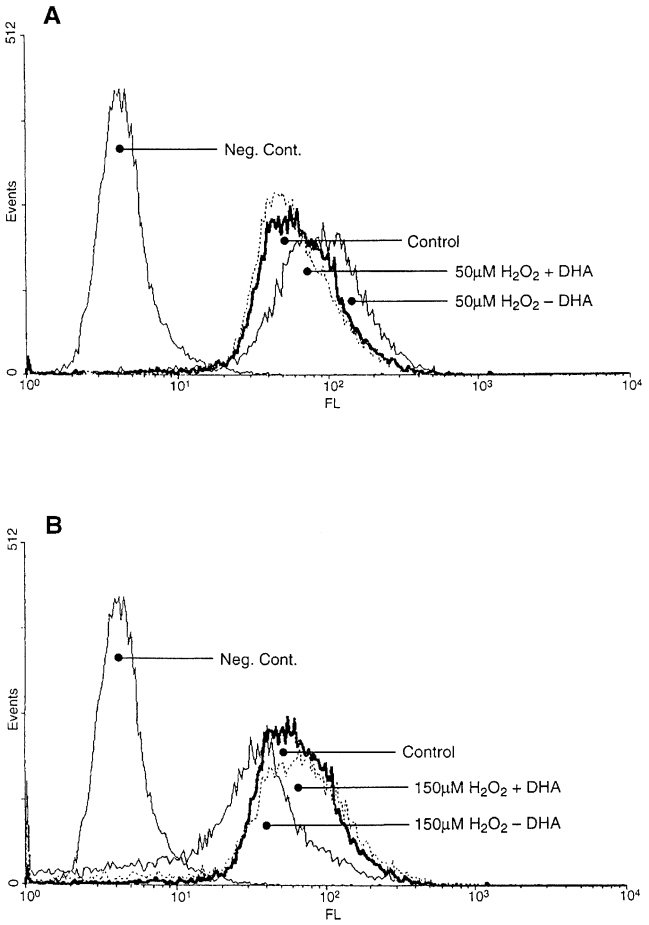


FIG. 6. Expression of Bcl-2 in cells exposed to H_2O_2. Effect of vitamin C. HL-60 cells were preincubated in buffer or 150 μM DHA for 15 min, washed and exposed for 6 hr to 50 μM H_2O_2 (A) or 150 μM H_2O_2 (B) and incubated for 24 hr. Cells were stained for Bcl-2 expression.

ated from oxygen metabolism [29]. Aerobic organisms are provided with well-balanced mechanisms that neutralize the oxidative effects of oxygen and its reactive metabolites [30]. Ascorbic acid is included among the radical trapping antioxidants and is considered one of the most efficient antioxidants [31]. Ascorbic acid concentrations in human plasma range from 20 to 200 μM, depending on dietary ascorbic acid and the time of sampling in relation to ingestion [32]. The concentration of ascorbic acid in human cells exceeds that in the blood, sometimes by an order of magnitude. For example, cells of the host defense system, such as neutrophils and monocytes, accumulate high (mM) intracellular concentrations of ascorbic acid [2, 33, 34].

Previous studies have shown that human neutrophils and HL-60 cells accumulate high concentrations of ascorbic acid by a complex mechanism involving the facilitated transport of DHA down a concentration gradient through facilitative glucose transporters, followed by the reduction of DHA to ascorbic acid and the intracellular trapping of ascorbic acid [3–5]. However, the fate of the intracellular accumulated ascorbic acid has not been as extensively investigated. In the present study, we report that a marked decrease in the intracellular concentration of ascorbic acid occurs in HL-60 cells preloaded with DHA upon their incubation for 4–8 hr in DHA-free medium. The decrease in ascorbic acid concentration may be a result of metabolic use of ascorbic acid by the cells, or partial oxidation of ascorbic acid to DHA, which is then exported outside the cells.

In the present study, we have found that loading of HL-60 cells with ascorbic acid provided strong protection from apoptosis associated with serum and glucose removal from the culture medium. Previous studies have shown that serum provides growth and survival factors and that its removal causes an oxidative stress that induces apoptosis in some cells [35]. Our findings are in line with Barroso *et al.*, who have shown that ascorbate and α-tocopherol can prevent lipid peroxidation and apoptosis caused by serum withdrawal when added to culture media of HL-60 and Daudi cells [35].

We also determined the intracellular peroxide and ascorbic acid pool following exposure of preloaded HL-60 cells with ascorbic acid to H_2O_2. A marked increase in peroxide concentrations, as measured by oxidation of DCFH to DCF and a correlated decrease in intracellular concentrations of ascorbic acid, was observed upon incubation of HL-60 cells with increasing concentrations of H_2O_2.

It has been reported that H_2O_2 can cross the cell membrane [36] and diffuse into intracellular compartments of HL-60 cells [37], where it can cause the oxidation of ascorbic acid to DHA, which is then transported outside the cells, or which can undergo irreversible breakdown into 2,3-diketo-L-gulonic acid, which then is broken down further.

We addressed the question as to whether ascorbic acid loading of HL-60 cells could reduce apoptosis induced due to oxidative stress. It is well documented that reactive oxygen species such as $O_2^{\cdot}$, H_2O_2, and $OH^{\cdot}$ are capable of damaging many cellular biomolecules, including DNA, and induce apoptosis. Exposure to low doses of H_2O_2 induces apoptosis in a variety of cell types [38]. Recently, it was shown that in myeloid leukemia cells one of the factors that can influence cell susceptibility to induction of apoptosis is the extent of intracellular oxidative stress. Cells with a higher intrinsic level of peroxide production showed a higher sensitivity to induction of apoptosis [39]. In our study, we have shown that exposure of HL-60 cells to increasing concentrations of H_2O_2 resulted in elevated intracellular concentrations of peroxides and increased apoptosis. In parallel experiments, we have shown that exposure of preloaded HL-60 cells with DHA to increasing concentrations of H_2O_2 resulted in reduced intracellular concentrations of ascorbic acid and decreased apoptosis. We speculate that ascorbic acid, being an antioxidant, partially neutralizes peroxides and decreases the intracellular oxidative level, and thus protects cells from oxidative-induced apoptosis.

Our results are consistent with previous studies that have

shown that antioxidants can reduce the extent of apoptosis induced by a variety of cytotoxic agents in different cell types [reviewed in 40, 41]. Antioxidants such as *N*-acetyl cysteine (NAC) or thioredoxin prevented apoptosis induced by the cytokine tumor necrosis factor α (TNF-α) [42–44]. The antioxidants ascorbic acid and *N*,*N*^{1}-diphenyl-*p*-phenylenediamine inhibited buthionine sulfoximine (BSO)-induced death of the hypothalamic neural cell line GTI-7 and reduced reactive oxygen species [16].

We show in the present study that exposure of HL-60 cells to low concentrations of H_2O_2 (50 μM), (a concentration that does not induce apoptosis in cells) resulted in a slight upregulated expression of Bcl-2. Bcl-2 is a survival factor and it protects cells against apoptosis induced by different stimuli, including reactive oxygen intermediates [15, 16]. Survival of cells exposed to oxidative stress is determined by both antioxidant capacity and the activities of various damage removal and repair enzymes in cells. It is possible that at the low H_2O_2 concentration (50 μM), apoptotic pathways were suboptimally triggered and that they activated Bcl-2 up-regulation as a protective mechanism. Upregulated expression of Bcl-2 was observed in additional cell lines exposed to low concentrations of H_2O_2 [18–20].

Previous studies performed with prokaryote and eukaryote cells have shown that exposure to low concentrations of hydrogen peroxide induces resistance to apoptotic stimuli [45–47]. The resistance is explained by the overexpression of several proteins, some of which are known as antioxidant enzymes [48]. Taken together, our and other findings, that low hydrogen peroxide concentrations enhance Bcl-2 expression and that low concentrations of hydrogen peroxide induce resistance in cells, lead one to speculate that the enhanced Bcl-2 expression induced in cells contributes to the resistance of the cells to subsequent apoptotic signals.

We have shown in our study that at higher H_2O_2 concentrations (150 μM), a concentration that increases the percentage of apoptotic cells up to 33.6%, a decrease in the expression of Bcl-2 was observed. This might be accounted for by depressed protein synthesis in cells exposed to intermediate concentrations of H_2O_2 [17] and by the inability of apoptotic cells to synthesize Bcl-2.

No significant variation of Bcl-2 expression was measured following exposure of HL-60 cells preincubated with DHA to 50 or 150 μM H_2O_2 (data not shown). The increased antioxidant capacity of ascorbic acid-loaded cells also protected the cells from a decrease in Bcl-2 expression following their exposure to 150 μM H_2O_2.

In summary, the results indicate that ascorbic acid loading provides strong protection from apoptosis associated with serum- and glucose-free culture and from apoptosis induced by oxidative stress. We speculate that ascorbic acid decreases the intracellular oxidative level in cells, thus enabling them to tolerate increased levels of oxidative stress before undergoing apoptosis.

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REFERENCES

1. Nishikimi M and Yagi K, Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. *Am J Clin Nutr* **54:** 1203S–1208S, 1991.
2. Washko P, Rotrosen D and Levine M, Ascorbic acid transport and accumulation in human neutrophils. *J Biol Chem* **264:** 18996–19002, 1989.
3. Welch RW, Wang Y, Crossman Jr. A, Park JB, Kirk K and Levine M, Accumulation of vitamin C (ascorbic acid) and its oxidized metabolite dehydroascorbic acid occurs by separate mechanisms. *J Biol Chem* **270:** 12584–12592, 1995.
4. Vera JC, Rivas CI, Zhang RH, Farber CM and Golde DW, Human HL-60 myeloid leukemia cells transport dehydroascorbic acid via the glucose transports and accumulate reduced ascorbic acid. *Blood* **84:** 1628–1634, 1994.
5. Vera JC, Rivas CI, Velasquez FV, Zhang RH, Concha II and Golde DW, Resolution of the facilitated transport of dehydroascorbic acid from its intracellular accumulation as ascorbic acid. *J Biol Chem* **270:** 23706–23712, 1995.
6. Crandon JH, Lund CC and Dill DB, Experimental human scurvy. *N Engl J Med* **223:** 353–369, 1940.
7. Englard S and Steifter SA, The biochemical functions of ascorbic acid. *Annu Rev Nutr* **6:** 365–406, 1986.
8. Padh H, Cellular functions of ascorbic acid. *Cell Biol* **68:** 1166–1173, 1990.
9. Washko P, Rotrosen D and Levine M, Ascorbic acid in human neutrophils. *Am J Clin Nutr* **54:** 1221S–1227S, 1991.
10. Byung PY, Cellular defenses against damage from reactive oxygen species. *Physiol Rev* **74:** 139–163, 1994.
11. Buettner GR, Ascorbic acid antioxidation in the presence of iron and copper chelates. *Free Rad Res Commun* **1:** 349–353, 1986.
12. Gardner A, Xu F-H, Fady C, Sarafian T, Tu Y and Lichtenstein A, Evidence against the hypothesis that Bcl-2 inhibits apoptosis through an anti-oxidant effect. *Cell Death Differ* **4:** 487–496, 1997.
13. Jacobson MD and Raff MC, Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* **374:** 814–816, 1995.
14. Yang-ja Lee and Shacter E, Bcl-2 does not protect Burkitt's lymphoma cells from oxidant-induced cell death. *Blood* **89:** 4480–4492, 1997.
15. Hockenbery DM, Olivai ZN, Yin XM, Milliman CL and Korsmeyer SJ, Bcl-2-functions in an anti-oxidant pathway to prevent apoptosis. *Cell* **75:** 241–251, 1993.
16. Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T and Bredesen DE, Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* **262:** 1274–1277, 1993.
17. Wiese AG, Pacifici RE and Davies KJA, Transient adaptation to oxidative stress mammalian cells. *Arch Biochem Biophys* **318:** 231–240, 1995.
18. Tu Y, Xu FH, Liu J, Vescio R, Berenson J, Fady C and Lichtenstein A, Upregulated expression of Bcl-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. *Blood* **88:** 1805–1812, 1996.
19. Bladier C, Wolvetang EJ, Hutchinson P, de Haan JB and Kola I, Response of a primary human fibroblast cell line to H_2O_2. Senescence-like growth arrest or apoptosis? *Cell Growth Diff* **8:** 589–598, 1997.
20. Hu ZB, Yang GS, Li M, Miyamoto N, Minden MD and McCulloch EA, Mechanism of cytosine arabinoside toxicity to the blast cells of acute myeloblastic leukemia: Involvement of free radicals. *Leukemia* **9:** 789–798, 1995.

21. Fabian I, Lass M, Kletter Y and Golde DW, Differentiation and functional activity of human eosinophilic cells from a human promyelocytic leukemia (HL-60) cell line: the response to six recombinant hematopoietic growth factors. *Blood* **80:** 788–794, 1992.
22. Kalir HH and Mytilineou C, Ascorbic acid in mesencephalic cultures: Effects on dopaminergic neuron development. *J Neurochem* **57:** 458–464, 1991.
23. Fernandes RS and Cotter TG, Apoptosis or necrosis: intracellular levels of glutathione influence mode of cell death. *Biochem Pharmacol* **48:** 675–681, 1994.
24. Gavrieli Y, Sherman Y and Ben-Sasson SA, Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119:** 490–501, 1992.
25. Smith CA, Williams GT, Kingston R, Jenkinson EJ and Owen JJ, Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* **337:** 181–184, 1989.
26. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC and Thomas M, Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* **130:** 1910–1917, 1983.
27. Washko P and Levine M, Inhibition of ascorbic acid transport in human neutrophils by glucose. *J Biol Chem* **267:** 23568–23574, 1992.
28. Verhaegen S, McGowan AJ, Brophy AR, Fernandes RS and Cotter TG, Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. *Biochem Pharmacol* **50:** 1021–1029, 1995.
29. Fridovich I, The biology of oxygen radicals. *Science* **201:** 875–880, 1978.
30. Davies KJA, Intracellular proteolytic systems may function as secondary antioxidant defenses: a hypothesis. *Free Rad Biol Med* **2:** 155–173, 1986.
31. Frei B, Stocker R, England I and Ames BN, Ascorbic acid: the most effective antioxidant in human blood plasma. *Adv Exp Med Biol* **264:** 155–163, 1990.
32. Liu TZ, Chin N, Kiser M and Bigler WN, Specific spectrophotometry of ascorbic acid in serum or plasma by use of ascorbic acid oxidase. *Clin Chem* **28:** 2225–2228, 1982.
33. Bergsten P, Amitai G, Kehrl J, Dhariwal KR, Klein HG and Levine M, Millimolar concentrations of ascorbic acid in purified human mononuclear leukocytes. Depletion and reaccumulation. *J Biol Chem* **265:** 2584–2587, 1990.
34. Washko PW, Wang Y and Levine M, Ascorbic acid recycling in human neutrophils. *J Biol Chem* **268:** 15531–15535, 1993.
35. Barroso MP, Gomez-Diaz C, Lopez-Lluch G, Malagon MM, Crane FL and Navas P, Ascorbate and α-tocopherol prevent apoptosis induced by serum removal independent of Bcl-2. *Arch Biochem Biophys* **343:** 243–248, 1997.
36. Cochrane CG, Schraufstatter IU, Hyslop PA and Jackson JH, Cellular and biochemical events in oxygen injury. In: *Oxyradicals in Molecular Biology and Pathology* (Eds. Cerutti PA, Fridovich I and McCord JM), pp. 125–136. Alan R. Liss Inc., New York, 1988.
37. Ohno Y and Gallin JI, Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. *J Biol Chem* **260:** 8438–8446, 1985.
38. Lennon SV, Martin SJ and Cotter TG, Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif* **24:** 203–214, 1991.
39. Lotem J, Peled-Kamar M, Groner Y and Sachs L, Cellular oxidative stress and the control of apoptosis by wild-type p53, cytotoxic compounds, and cytokines. *Proc Natl Acad Sci USA* **93:** 9166–9171, 1996.
40. Buttke TM and Sandstrom PA, Oxidative stress as a mediator of apoptosis. *Immunol Today* **15:** 7–10, 1994.
41. Slater AF, Nobel CS and Orrenius S, The role of intracellular oxidants in apoptosis. *Biochim Biophys Acta* **1271:** 59–62, 1995.
42. Chang DJ, Ringold GM and Heller RA, Cell killing and induction of manganous superoxide dismutase by tumor necrosis factor-alpha is mediated by lipoxygenase metabolites of arachidonic acid. *Biochem Biophys Res Commun* **188:** 538–546, 1992.
43. Malorni W, Rivabene R, Santini MT and Donelli G, *N*-acetylcysteine inhibits apoptosis and decreases viral particles in HIV chronically infected U937 cells. *FEBS Lett* **327:** 75–78, 1993.
44. Matsuda M, Masutani H, Nakamura H, Miyajima S, Yamauchi A, Yonehara S, Uchida A, Irimajiri K, Horiuchi A and Yodoi J, Protective activity of adult T cell leukemia-derived factor (ADF) against tumor necrosis factor-dependent cytotoxicity on U937 cells. *J Immunol* **147:** 3837–3841, 1991.
45. Spitz DR, Dewey WC and Li GC, Hydrogen peroxide or heat shock induces resistance to hydrogen peroxide in Chinese hamster fibroblasts. *J Cell Physiol* **131:** 364–373, 1987.
46. Christman MF, Morgan RW, Jacobson FS and Ames BN, Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41:** 753–762, 1985.
47. Maellaro E, Del Bello B and Comporti M, Protection by ascorbate against apoptosis of thymocytes: implications of ascorbate-induced nonlethal oxidative stress and poly(ADP-ribosyl)ation. *Exp Cell Res* **226:** 105–113, 1996.
48. Keyse SM and Tyrrell RM, Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide and sodium arsenite. *Proc Natl Acad Sci USA* **86:** 99–103, 1989.