Supplemental Ascorbate or α-Tocopherol Induces Cell Death in Cu-Deficient HL-60 Cells

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Cytochrome *c* oxidase (CCO) is the Cu-dependent, terminal respiratory complex of the mitochondrial electron transport chain. Inhibition of CCO can promote oxidative stress by increasing mitochondrial production of reactive oxygen species (ROS). Because mitochondria have an important role in apoptosis as both a target and source for ROS, enhanced ROS production resulting from inhibition of CCO by Cu deficiency may trigger apoptosis. The present study focuses on the mitochondrial effects of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET), which inhibits CCO by causing cellular Cu deficiency, and the antioxidants ascorbate and α-tocopherol in a human promyelocytic leukemia cell line (HL-60). The following effects were observed: (i) TET reduced both cell growth and viability only in the presence of ascorbate or α-tocopherol; (ii) TET reduced CCO activity and increased mitochondrial ROS production as indicated by increased expression of Mn superoxide dismutase, but the induction of Mn superoxide dismutase was not affected by ascorbate or α-tocopherol; (iii) TET acted independently of ascorbate or α-tocopherol in disrupting mitochondrial membrane potential; (iv) TET did not increase caspase-8 activity in the absence of ascorbate or α-tocopherol; and (v) TET did not increase transfer of cytochrome *c* from mitochondria to the cytosol unless α-tocopherol was present. These findings indicate that reduction in CCO activity by TET-induced Cu deficiency increased oxidative stress in HL-60 cells sufficiently to disrupt the electrochemical gradient of the inner mitochondrial membrane but did not trigger cell death. Also, ascorbate and α-tocopherol did not alleviate oxidative stress but may have become pro-oxidants, adding to the oxidant burden sufficiently to trigger cell death in TET-treated cells. Exp Biol Med 229:885–894, 2004

Key Words: copper deficiency; oxidative stress; HL-60 cells; cell death; ascorbate; α-tocopherol

Synthesis of ATP by mitochondria depends on energy derived from the flow of electrons from metabolic intermediates through the electron transport chain to molecular oxygen. Although the electron transport chain converts about 85%–90% of the oxygen utilized by cells to water, 1%–2% of the oxygen is converted to reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (1–3). However, mitochondrially generated ROS normally cause little oxidative damage because mitochondria possess an efficient antioxidant defense system composed of Mn superoxide dismutase (MnSOD), glutathione peroxidase, NADPH transhydrogenase, reduced glutathione, NADPH, and vitamins E and C (4–6).

Perturbations in the electron transport chain can lead to oxidative stress by creating an imbalance between mitochondrial ROS production and antioxidant defenses. Production of ROS by mitochondria is largely determined by the redox state of the electron chain and is highest when the respiratory complexes are highly reduced (1). Thus, blockage of electron flow near the terminal end of the electron transport chain increases the reducing potential of the respiratory complexes upstream from the blockage and causes an increase in ROS production as single electrons are transferred to molecular oxygen. This principle has been demonstrated with mitochondria from the flight muscles of house flies, in which partial inhibition of cytochrome *c* oxidase (CCO), which catalyzes the terminal reaction of the electron transport chain, increases the rate of mitochondrial hydrogen peroxide production (7).

CCO is a Cu-dependent enzyme whose activity in a

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variety of tissues is inhibited by Cu deficiency (8–10). Although the physiological or pathological relevance of CCO inhibition during Cu deficiency is not clear, increased mitochondrial ROS production and oxidative damage are potential outcomes, based on the finding that partial inhibition of CCO enhances the rate of mitochondrial hydrogen peroxide production. This hypothesis is supported by results obtained from HL-60 cells cultured in serum-free medium in the absence of supplemental Cu. When HL-60 cells are cultured under these conditions, CCO activity is reduced, and the reduction in activity is accompanied by increases in MnSOD content and carbonyl content in some mitochondrial proteins (11). Given that MnSOD is induced by hydrogen peroxide and other oxidants (12, 13) and that protein carbonyls are indicators of ROS-induced protein oxidation (14), these findings are consistent with increased oxidative stress and damage to mitochondria in the Cu-deficient cells.

The original intent of the present study was to determine whether ascorbic acid and α-tocopherol could prevent or lessen the degree of oxidative stress associated with cellular Cu deficiency. However, early in the course of the study, it was found that these antioxidants decreased the growth and viability of Cu-deficient HL-60 cells. Accordingly, the present study had two objectives: (i) to determine whether ascorbate and α-tocopherol serve as antioxidants in Cu-deficient HL-60 cells by examining their effect on MnSOD expression and (ii) to propose a possible mechanism for the negative effects of ascorbate and α-tocopherol on the growth and viability of Cu-deficient HL-60 cells.

Materials and Methods

Cell Culture. HL-60 cells, a human promyelocytic cell line, were purchased from the American Type Culture Collection (Rockville, MD). Stock cultures were maintained in Iscove's modified Delbecco's medium (IMDD) supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) in spinner flasks (36°C, 5% CO_2, 93%–95% humidity). Cells were transferred to T150 flasks to obtain a density of 2.5–3 × 10^5 cells/ml in IMDD containing 5% fetal bovine serum and either 20 μ*M* of the Cu chelator *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET) or no TET. Cells were allowed to grow for 5 days and were then divided into three groups. One group of cells received no additions, whereas either ascorbate (0.5 m*M* final concentration) or α-tocopherol acetate (0.1 m*M* final concentration) was added to the other groups. After antioxidant addition, the cells were allowed to grow for various times depending on experimental needs. Cell viability was routinely assessed by trypan blue exclusion. Trace metal content, CCO activity, the expression of MnSOD, and cytosolic cytochrome *c* content were determined 24 hrs after antioxidant addition. Mitochondrial membrane potential ($\Delta\psi_m$) and caspase-8 activity were measured 2, 4, 6, and 24 hrs after antioxidant addition.

CCO Activity. The HL-60 cells were homogenized by rupturing the cell membranes using nitrogen decompression (11). Homogenates from 2 × 10^7 cells were sonicated (Model W380; Heat Systems-Ultrasonics, Inc., Farmingdale, NY), and CCO activity was assayed by measuring the decrease in optical density of ferrocytochrome *c* as previously described (15).

Metal Analysis. Duplicate aliquots of cell suspension were pipetted into acid-washed polypropylene tubes and centrifuged at 200 *g* for 10 mins. The cells were then resuspended and washed twice in 5.0 ml of phosphate-buffered saline (PBS) containing 0.14 *M* NaCl, 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, and 2.7 m*M* KCl, pH 7.4. After final resuspension in 5.0 ml of PBS, the cells were counted and then centrifuged at 750 *g* for 10 mins. The resulting pellet was dissolved by adding 0.5 ml of ultrapure, concentrated nitric acid (Instra-Analyzed Reagent; Mallinckrodt Baker, Inc., Phillipsburg, NJ) and incubating the capped tubes overnight at 37°C. Copper, iron, zinc, and manganese were determined by inductively coupled argon plasma spectrometry (Optima 3300DV spectrometer; Perkin Elmer Corp., Norwalk, CT).

MnSOD Protein and mRNA Content. Approximately 10^7 cells were suspended in cold buffer containing 0.25 *M* sucrose, 0.001 *M* EDTA, and 0.01 *M* Tris-HCl, pH 7.6. After pressurizing the cell suspension under nitrogen for 5 mins at 300 psi in a cell-disruption bomb (Parr Instrument Co., Moline, IL), the cell membranes were ruptured by rapid decompression. Cellular debris was removed by centrifugation at 750 *g* for 5 mins. MnSOD content of the supernatant was determined by immunoblotting as previously described (11) using a modified blocking buffer containing 137 m*M* NaCl, 2.7 m*M* KCl, 10.1 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, 0.05% Tween 20, 1% bovine serum albumin, pH 7.4 (BSA/PBS-T), and rabbit anti-MnSOD polyclonal antibody (Stressgen Biotechnologies, Victoria, British Columbia, Canada) diluted 1:5000 in BSA/PBS-T. Visualization of MnSOD was accomplished by chemiluminescence and exposure of the blots to luminescence detection film (ECL Western blotting detection reagents and Hyperfilm-ECL; Amersham Biosciences, Piscataway, NJ). Immunoreactive bands were analyzed by imaging densitometry (GS-700 Imaging Densitometer; Bio-Rad Laboratories, Hercules, CA).

Total cellular RNA was isolated by phenol–guanidine thiocyanate–chloroform extraction (16). Both the quality and quantity of RNA were verified by gel electrophoresis and staining with ethidium bromide. The relative mRNA levels for MnSOD were determined by reverse transcriptase–polymerase chain reaction (RT-PCR) using the Access RT-PCR system (Promega, Madison, WI) with Quantum 18S RNA internal standard (Ambion, Austin, TX). The PCR reaction mixture (50 μl) contained 0.2 m*M* dNPT, 1.0 m*M* $MgSO_4$, 1 × avian myoblastosis virus/

Thermus flavus (AMV/Tfl) reaction buffer, 1 μ*M* of each forward and reverse primer, 0.1 U of AMV reverse transcriptase, 0.1 U of *Tfl* reverse transcriptase, 4 μl of 18S rRNA at a 1:9 primer:competimer ratio, and 0.5 μg of RNA template. PCR was performed on a PTC-100 Programmable Thermal Controller (MJ Research, Incline Village, NV). Primer pairs were designed (Omiga Software, Genetics Computer Group, Madison, WI) for amplification of cDNA corresponding to bases 265–864 of MnSOD mRNA (GeneBank accession No. Y00985). Primers, synthesized by Gibco BRL Custom Primer (Life Technologies, Rockville, MD), were as follows: forward, 5′-CTACGTGAACAACCTGAACG-3′; and reverse, 5′-AGAATGCTACAATAGAGCAGC-3′. The reaction was performed as follows: 1 cycle for reverse transcription at 42°C for 45 mins; 1 cycle for AMV reverse transcriptase inactivation and RNA/cDNA/primer denaturation at 94°C for 2 mins; and 26 cycles for second-strand cDNA synthesis and PCR amplification with denaturation at 94°C for 15 secs, annealing at 55°C for 15 secs, and extension at 68°C for 90 secs. A final extension cycle was performed at 72°C for 7 mins, and the final incubation temperature was kept at 4°C. PCR products were incubated 1:10,000 with Vistra Green for 15 mins before electrophoresis on 4% polyacrylamide gels. The fluorescence of the endogenous target and 18S standards was quantified with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results are expressed as the ratio of sample:internal standard for each sample.

Mitochondrial Membrane Potential. $\Delta\psi_m$ was estimated by measuring the aggregation of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (DePsipher) resulting from membrane polarization with a DePsipher assay kit (R&D Systems, Inc., Minneapolis, MN). Following the manufacturer's protocol, 1×10^6 cells were suspended in 1 ml of DePsipher solution (5 μg/ml) and incubated in 5% CO_2 at 37°C for 20 mins. After washing twice with PBS, the cells were analyzed by flow cytometry (Epics X2-MCL flow cytometer; Beckman-Coulter, Inc., Miami, FL) using a single laser emitting excitation light at 488 nm. Membrane polarization was determined by the shift in fluorescence emission from 530 nm (monometric DePsipher) to 590 nm (DePsipher aggregate).

Caspase-8 Assay. Caspase-8 activity was measured using the ApoAlert Caspase-8 colorimetric assay kit (Clonetech Laboratories, Inc., Palo Alto, CA). According to the manufacturer's protocol, 2×10^6 cells were centrifuged at 400 *g* for 5 mins and then resuspended in 50 μl of cold lysis buffer. Cellular debris was removed from the lysates by centrifugation, and the supernatants (50 μl) were transferred to clean microcentrifuge tubes and mixed with an equal volume of 2 × reaction buffer. The mixtures were incubated with Ile-Glu-Asp-Thr-7–amino-4-trifluoromethyl coumarin (IETD-AFC)–conjugated substrate for 1 hr at 37°C. After incubation, fluorescence emission of each sample was measured at 505 nm with excitation at 400 nm.

Cytochrome *c* Release. Cells (2×10^6) were suspended in 50 μl of cold (4°C) homogenizing buffer (20 m*M* HEPES, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulfonyl fluoride, and 250 m*M* sucrose, pH 7.5) and homogenized with a glass microhomogenizer. The homogenates were centrifuged at 750 *g* for 10 mins at 4°C to remove cellular debris. Mitochondria were isolated by centrifuging the supernatant at 10,000 *g* for 15 mins. The resulting mitochondrial pellets were washed with homogenizing buffer before final resuspension in 50 μl of the same buffer. The cytosolic fraction was prepared from the 10,000-*g* supernatant by centrifugation at 100,000 *g* for 1 hr. Cytosolic and mitochondrial proteins were separated by electrophoresis and electroblotted as described above (see MnSOD protein and mRNA content). The cytosolic and mitochondrial fractions were also probed with anti-MnSOD antibody. MnSOD was found predominately in the mitochondrial fraction, indicating that the presence of cytochrome *c* in the cytoplasmic fraction was not due to mitochondrial rupture during organelle isolation. Cytosolic and mitochondrial cytochrome *c* contents were detected by probing the blotted proteins with anti-cytochrome *c* monoclonal antibody (clone 6H2.B4; Promega Corp., Madison, WI) and horseradish peroxidase–coupled anti-mouse immunoglobulin G (Pierce Biotechnology, Inc., Rockford, IL). Visualization of cytochrome *c* was accomplished by chemiluminescence and exposure of the blots to luminescence detection film (ECL Western blotting detection reagents and Hyperfilm-ECL; Amersham Biosciences, Piscataway, NJ). Immunoreactive bands were analyzed by imaging densitometry (GS-700 Imaging Densitometer; Bio-Rad Laboratories, Hercules, CA), and cytochrome *c* release from mitochondria was determined as the ratio of cytochrome *c* present in the cytosolic fraction to the total present in the cytosol and mitochondria.

Data Analysis. Effects of TET, ascorbate, α-tocopherol, and time were analyzed by two-way or three-way ANOVA. Bonferroni contrasts were used for *post hoc* comparisons between individual means for data showing significant interaction (SAS/STAT 8.02; SAS Institute, Inc., Cary, NC).

Results

As shown in Figure 1, treatment of HL-60 cells with 20 μ*M* TET retarded their growth by approximately 40%. However, the viability of TET-treated and untreated cells was similar and remained stable at 85%–95% from the fourth day to the twelfth day after cultures were initiated. In the absence of TET, the addition of ascorbate (0.5 m*M* final concentration) or α-tocopherol (0.1 m*M* final concentration) to the medium on the fifth day after cultures were initiated had little effect on cell growth or viability. In contrast, the

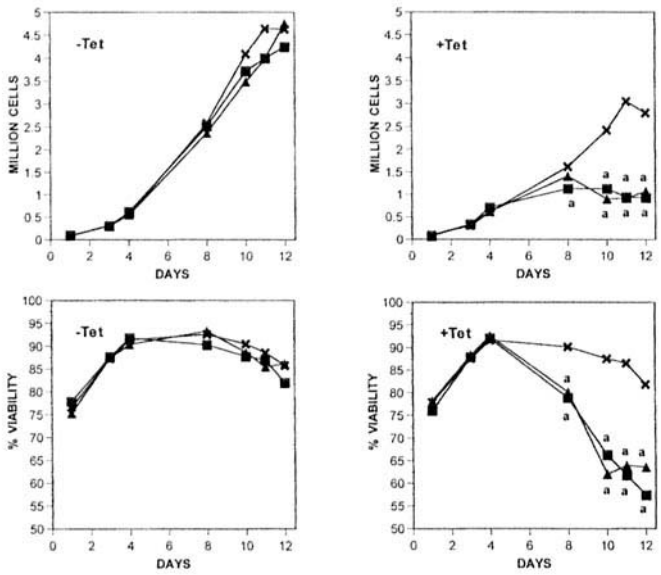

Figure 1. Effect of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET), ascorbate, and α-tocopherol on the growth and viability of HL-60 cells. Cells were grown in the absence (–TET) or presence (+TET) of 20 μ*M* TET. After 5 days of growth, the cultures received either no additional treatment (×) or addition of 0.5 m*M* ascorbate (▲) or 0.1 m*M* α-tocopherol (■). Each point represents the mean obtained from cell counts and viability measurements for duplicate samples of cells from three flasks. Growth and viability were both affected by a significant three-way interaction between TET, antioxidant treatment, and day ($P < 0.05$, ANOVA). For clarity, error bars are not shown; the pooled standard deviations are 0.022 and 2.34 for cell number and viability, respectively. Comparison of individual means for cell counts and viability for TET-treated and untreated cells grown in the absence of antioxidants showed that TET treatment significantly reduced the number of cells at days 8–12 ($P < 0.05$, Bonferroni contrasts) but did not affect cell viability on these days ($P > 0.05$, Bonferroni contrasts). Means for cell number and viability of TET-treated cells grown in the presence of antioxidants are labeled with an "a" if they were significantly lower than those of TET-treated cells grown in the absence of antioxidants ($P < 0.05$, Bonferroni contrasts).

growth and viability of TET-treated cells were severely reduced by the addition of ascorbate or α-tocopherol.

Table 1 shows that Cu concentrations were decreased by at least 50% in HL-60 cells grown for 5 days in medium containing 20 μ*M* TET. However, Cu concentrations were affected by an interaction between TET and antioxidant treatments ($P = 0.02$ for TET × antioxidant, ANOVA) that arose from a slight, but insignificant ($P > 0.05$), increase in Cu concentration caused by ascorbate in cells not treated with TET. Iron concentrations also were affected by an interaction between TET and antioxidant treatments ($P = 0.008$ for TET × antioxidant, ANOVA). Although TET treatment did not generally affect iron concentrations, α-tocopherol caused a slight ($P < 0.05$) increase in the iron concentration of TET-treated cells. Also, Mn concentrations were slightly lowered ($P < 0.05$, Bonferroni contrasts) by TET treatment except in cells exposed to α-tocopherol. Neither TET nor antioxidant treatment affected cellular zinc concentrations (data not shown).

As shown in Figure 2, TET treatment caused a marked reduction in CCO activity in HL-60 cells ($P < 0.0001$, ANOVA). Over all cells, CCO activity was 15.5 ± 1.35 U/mg protein in TET-treated cells and 52.6 ± 1.35 U/mg protein in cells not treated with TET. Exposure to ascorbate or α-tocopherol did not affect CCO activity in either untreated or TET-treated cells.

Figure 3 shows that TET treatment increased MnSOD expression in HL-60 cells. Immunoreactive MnSOD protein and MnSOD mRNA were both affected by a significant interaction between TET and antioxidant treatment ($P < 0.05$ for TET × antioxidant, ANOVA). This interaction occurred because MnSOD protein and mRNA were slightly increased in cells exposed to α-tocopherol but not treated with TET. However, comparisons of individual means indicated that the level of MnSOD protein and mRNA in these cells was not different ($P > 0.05$) from the levels either in cells not treated with TET or in cells not treated with TET but exposed to ascorbate. However, TET treatment significantly ($P < 0.05$) increased MnSOD protein levels in cells that received no exposure to antioxidants and in cells exposed to either ascorbate or α-tocopherol. TET treatment also significantly ($P < 0.05$) increased MnSOD mRNA levels in cells exposed to ascorbate. Although MnSOD mRNA appeared to be higher in TET-treated cells exposed to α-tocopherol than in

Table 1. Effects of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET) and Antioxidant Treatment on Copper, Iron, and Manganese Concentrations in HL-60 Cells[a]

	Cu concentration (ng/10^6 cells)		Fe concentration (ng/10^6 cells)		Mn concentration (ng/10^6 cells)	
Antioxidant	–TET	+TET	–TET	+TET	–TET	+TET
None	1.09 ± 0.05[b]	0.51 ± 0.06[c]	8.70 ± 0.10[b]	8.26 ± 0.17[b]	0.98±0.04[b,c]	0.74±0.03[d]
Ascorbate	1.34 ± 0.003[b]	0.30 ± 0.01[c]	8.51 ± 0.33[b]	7.83 ± 0.28[b]	1.03±0.04[b]	0.81±0.02[d,e]
α-Tocopherol	1.00 ± 0.002[b]	0.53 ± 0.08[c]	8.43 ± 0.62[b]	11.7 ± 0.78[c]	0.92±0.03[b,d]	0.89±0.04[b,d]
ANOVA				*P*		
TET	<0.0001		0.10		0.001	
Antioxidant	0.53		0.01		0.25	
TET × Antioxidant	0.02		0.008		0.04	

[a] Values are mean ± SEM for cells from two separate flasks.
[b–e] Means not sharing a common superscript are significantly different ($P < 0.05$, Tukey's test).

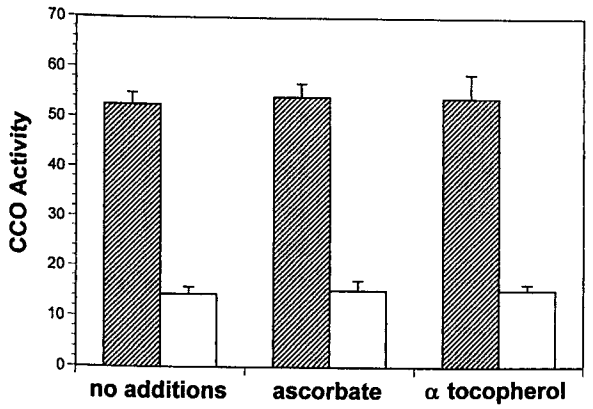

Figure 2. Effect of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET), ascorbate, and α-tocopherol on the cytochrome *c* oxidase (CCO) activity in HL-60 cells. CCO activity was measured in cells grown in either the absence (cross-hatched bar) or presence (open bar) of 20 μ*M* TET for 5 days with either no added antioxidant or 24 hours after the addition of 0.5 m*M* ascorbate or 0.1 m*M* α-tocopherol. CCO activity is expressed as units/mg protein, where 1 unit of activity is the amount of enzyme that oxidizes 1 nmole of ferrocytochrome *c*/min at 30°C. Values are mean ± SEM for measurements from three separate cultures. TET significantly lowered CCO activity ($P < 0.05$, ANOVA). Addition of antioxidants had no effect on activity ($P > 0.05$, ANOVA), and the TET × antioxidant interaction was not significant ($P > 0.05$, ANOVA).

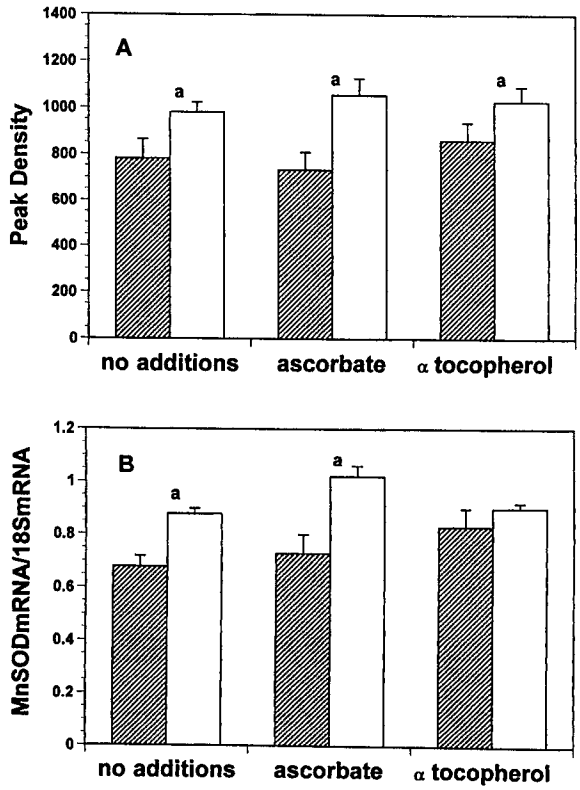

Figure 3. Effect of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET), ascorbate, and α-tocopherol on the expression of manganese superoxide dismutase (MnSOD). Densities of peaks representing immunoreactive MnSOD (A) and MnSOD mRNA/18S mRNA ratios (B) in cells grown in either the absence (cross-hatched bar) or presence (open bar) of 20 μ*M* TET for 5 days with either no added antioxidant or 24 hours after the addition of 0.5 m*M* ascorbate or 0.1 m*M* α-tocopherol. Values are mean ± SEM for measurements from three separate cultures. Immunoreactive MnSOD and MnSOD mRNA were both affected by a significant TET × antioxidant interaction ($P < 0.05$, ANOVA). Bars labeled with an "a" indicate that the means were higher in TET-treated cells compared with untreated cells ($P < 0.05$, Bonferroni contrasts).

untreated cells exposed to α-tocopherol, the difference was not statistically significant ($P > 0.05$).

Flow cytometry indicated that $\Delta\psi_m$ was affected by TET treatment and exposure to antioxidants. As shown in Figure 4, TET treatment caused an overall reduction in $\Delta\psi_m$ independently of ascorbate or α-tocopherol in cells sampled 2, 4, 6, and 24 hrs after exposure to the antioxidants ($P = 0.0002$ for the effect of TET, ANOVA). Over all TET-treated cells, $\Delta\psi_m$ was 120 ± 3 mean fluorescence units/10^3 events compared with 159 ± 3 mean fluorescence units/10^3 events in untreated cells. Exposure of the cells to ascorbate and α-tocopherol also lowered $\Delta\psi_m$ independently of TET treatment ($P = 0.009$ for the effect of antioxidants, ANOVA). Over all TET-treated and untreated cells, $\Delta\psi_m$ was 157 ± 4 mean fluorescence units/10^3 events in cells grown in the absence of antioxidants compared with 135 ± 4 mean fluorescence units/10^3 events and 124 ± 4 mean fluorescence units/10^3 events in cells exposed to ascorbate and α-tocopherol, respectively.

Treatment of HL-60 cells with TET had little effect on the activity of caspase-8 unless ascorbate or α-tocopherol was present in the medium. As shown in Figure 5, TET-treated cells tended to have higher caspase-8 activity than did untreated cells in the absence of ascorbate or α-tocopherol, but the difference was not significant ($P > 0.05$). However, caspase-8 was higher in TET-treated cells at 4, 6, and 24 hrs after exposure to ascorbate or α-tocopherol compared with the activity in TET-treated cells that were not exposed to the antioxidants ($P < 0.05$). Caspase-8 activity also was significantly higher in TET-treated cells compared with untreated cells at 4, 6, and 24 hrs after exposure to ascorbate or α-tocopherol ($P < 0.05$).

Western blot analysis was used to examine the effects of ascorbate and α-tocopherol translocation of cytochrome *c* to the cytosol in TET-treated and untreated cells. As shown in Figure 6, TET treatment did not significantly affect cytosolic cytochrome *c* in the absence of antioxidants or after 24 hrs of exposure to ascorbate. However, cytosolic cytochrome *c* was significantly ($P < 0.05$) elevated in TET-treated cells exposed to α-tocopherol for 24 hrs.

Discussion

Although TET treatment caused slight changes in the cellular concentrations of iron and Mn, the major effects of this Cu chelator on HL-60 cells were growth retardation and reduction in cellular Cu concentration with an accompanying reduction in the activity of CCO, a Cu-dependent enzyme. These findings are consistent with results from a previous study showing that treatment with Cu chelators can

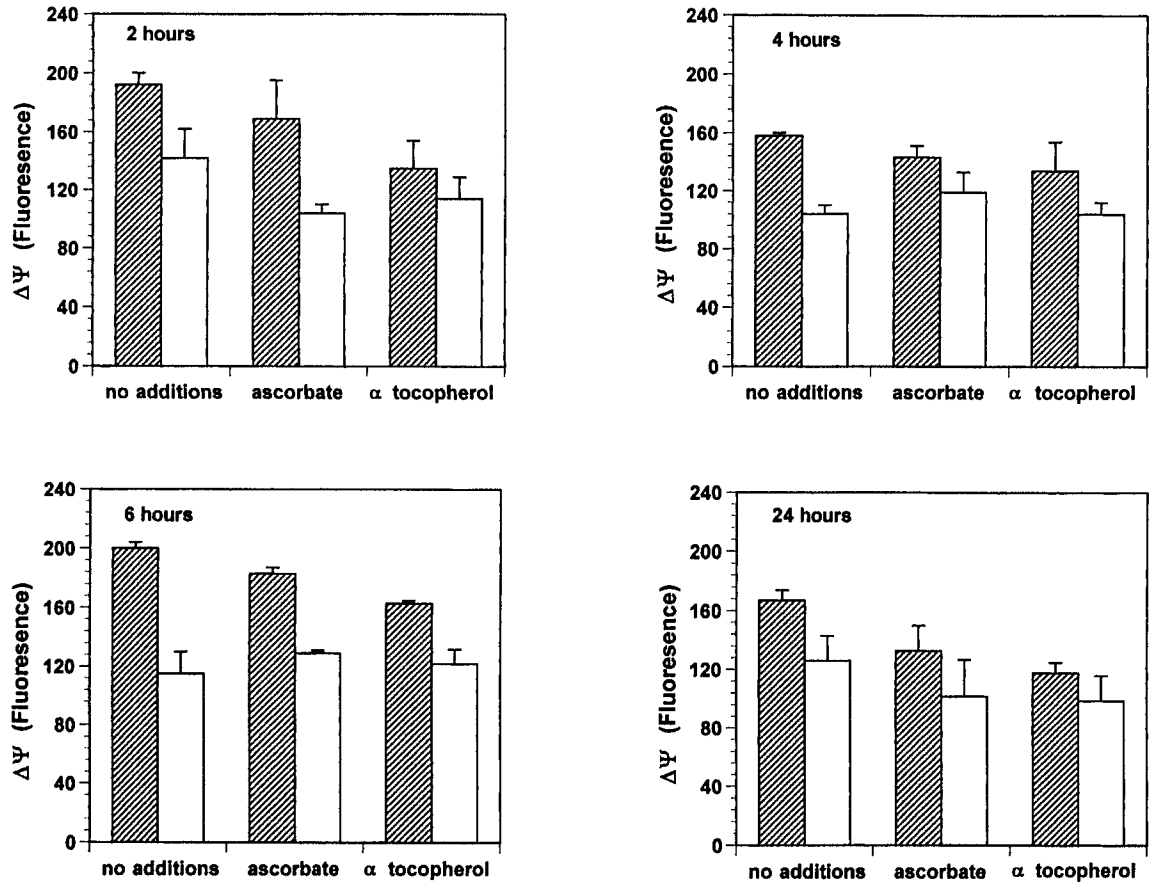

Figure 4. Effect of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET), ascorbate, and α-tocopherol on mitochondrial membrane potential. Mitochondrial membrane potential was assessed in cells grown in either the absence (cross-hatched bar) or presence (open bar) of 20 μ*M* TET for 5 days with either no added antioxidant or at 2, 4, 6, or 24 hrs after the addition of 0.5 m*M* ascorbate or 0.1 m*M* α-tocopherol. Values are mean ± SEM for measurements from three separate cultures. Membrane potential is shown as the fluorescence intensity resulting from the aggregation of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide upon membrane polarization. The membrane potentials were significantly affected by TET and antioxidants ($P < 0.05$, ANOVA), but the TET × antioxidant interaction was not significant ($P > 0.05$, ANOVA).

produce functional Cu deficiency in HL-60 cells (17). Metabolic perturbations resulting from cellular Cu deficiency most likely contributed to the reduction in the growth rate of HL-60 cells treated with TET. However, these metabolic perturbations were not sufficient to affect the viability of the TET-treated cells unless the cells were also treated with ascorbate or α-tocopherol. The negative effect of ascorbate and α-tocopherol on the viability of TET-treated cells was also reflected in the severe reductions in the growth rate of these cells.

In addition to indicating that TET-treated cells were Cu deficient, the reduction in CCO activity may have functional consequences related to oxidative stress. Inhibition of respiratory complexes can increase mitochondrial ROS production (1), and it has been shown that partial inhibition of CCO, the terminal respiratory complex in the electron transport chain, increases mitochondrial hydrogen peroxide generation (7). Although an increase in mitochondrial oxidant production in TET-treated cells was not measured directly in our study, increased expression of MnSOD suggests that an increase in mitochondrial hydrogen peroxide or other ROS production may have occurred. Our finding that TET treatment increased MnSOD expression in HL-60 cells is consistent with findings from previous studies showing that Cu deficiency in rats (18, 19) and cultured cells (11, 20) increases MnSOD activity, protein, and mRNA levels. The expression of MnSOD, a mitochondrial matrix enzyme involved in detoxifying superoxide, is increased by hydrogen peroxide and other ROS (12, 13). Because of its close proximity to the electron transport chain, a major source of intracellular ROS, induction of MnSOD may be particularly sensitive to intracellular oxidants generated by mitochondria. However, CCO is not the only Cu-dependent enzyme whose activity is inhibited by cellular Cu deficiency. Previous studies have shown that Cu,ZnSOD activity is also reduced in cells treated with Cu chelators (17, 20) or grown in Cu-deficient, serum-free medium (15). Although Cu,ZnSOD activity was not measured in the present study, its activity was most likely reduced because the TET-treated cells were Cu deficient, based on Cu content and CCO activity. Thus, the induction of MnSOD that we observed in TET-treated HL-60 cells

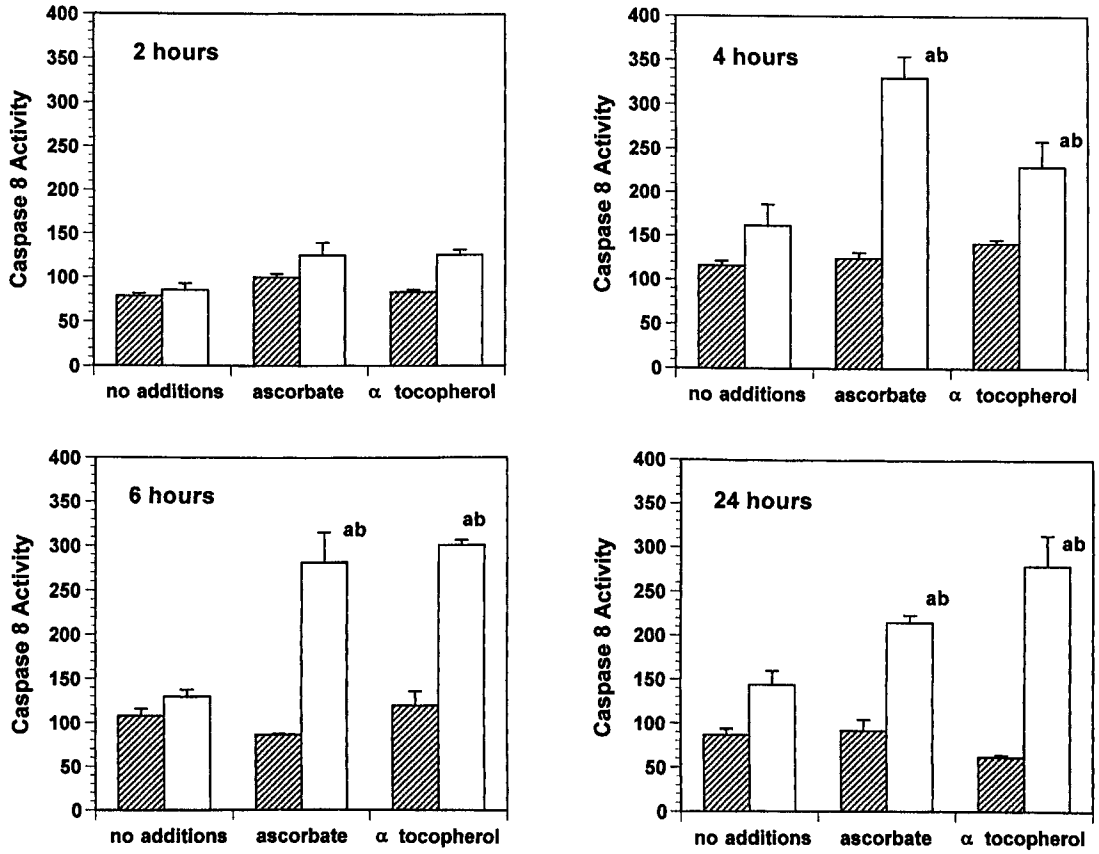

Figure 5. Effect of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET), ascorbate, and α-tocopherol on caspase-8 activity. Activity was measured in cells grown in either the absence (cross-hatched bar) or presence (open bar) of 20 μ*M* TET for 5 days with either no added antioxidant or at 2, 4, 6, or 24 hrs after the addition of 0.5 m*M* ascorbate or 0.1 m*M* α-tocopherol. Values are mean ± SEM for measurements from three separate cultures. Caspase-8 activity is the fluorescence of Ile-Glu-Asp-Thr-7-amino-4-trifluoromethyl coumarin substrate per 2×10^6 cells after 1 hr of incubation at 37°C. The activity was significantly affected by the TET × antioxidant interaction ($P < 0.05$, ANOVA). Bars labeled with an "a" indicate that the mean activity was higher in TET-treated cells compared with untreated cells. Bars labeled with a "b" indicate that the mean activity was higher in TET-treated cells exposed to antioxidant compared with TET-treated cells with no added antioxidant ($P < 0.05$, Bonferroni contrasts).

may be a consequence of both increased mitochondrial ROS production, resulting from the inhibition of CCO caused by Cu deficiency, and a general increase in cellular oxidative stress, resulting from inhibition of Cu,ZnSOD and impaired scavenging of superoxide radical.

ROS generated by inefficiency in the mitochondrial electron transport chain during Cu deficiency are potentially damaging to the mitochondria. In particular, the (4Fe-4S) iron-sulfur centers present in mitochondrial aconitase, succinate dehydrogenase, and respiratory complex I are susceptible to damage by superoxide (3). Hydrogen peroxide formed from superoxide by MnSOD in mitochondria can react with unscavenged superoxide to form highly reactive hydroxyl radical through the Haber-Weiss reaction catalyzed by mitochondrial iron (1). Hydroxyl radical may also be formed directly from hydrogen peroxide by the Fenton reaction in the presence of mitochondrial ferrous and cuprous ions (3). Under normal circumstances, oxidative damage to mitochondria resulting from ROS generated by the electron transport chain is controlled through an efficient antioxidant system composed of MnSOD, glutathione peroxidase, NADPH transhydrogenase, reduced glutathione, NADPH, and vitamins E and C (4–6). However, the decrease in $\Delta\psi_m$ that we observed in TET-treated cells suggests that Cu deficiency creates an imbalance between mitochondrial ROS generation and antioxidant defenses that produces sufficient oxidative stress to damage mitochondria and disrupt the electrochemical gradient of the inner membrane.

Mitochondria have a central, functional role in coordinating apoptosis through the release of soluble proteins from within the mitochondria to the cytosol, where they participate in the programmed destruction of the cell (21–23). Release of these proteins from mitochondria can be mediated by ROS and is increased as a result of oxidative stress (3, 24). The disruption of $\Delta\psi_m$ is an early event in the mitochondrial regulation of apoptosis and may be accompanied by translocation of cytochrome *c* from the inner mitochondrial membrane to the cytosol, where it participates in the initial steps of apoptosis (3, 25). Pro-caspase-8 is also

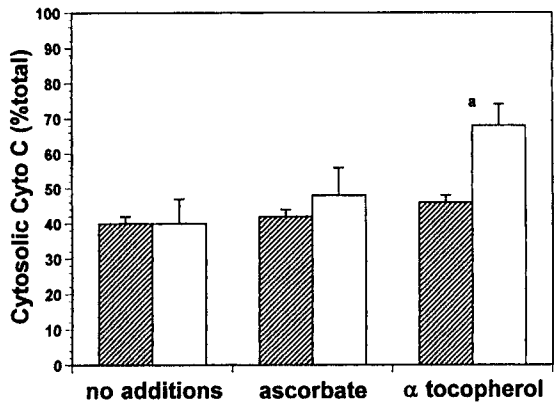

Figure 6. Effect of *N,N'*-bis(2-aminoethyl)-1,3-propanediamine (TET), ascorbate, and α-tocopherol on the content of cytochrome *c* in the cytosolic fraction. The percentage of total immunoreactive cytochrome *c* present in the cytosol of HL-60 cells was measured in cells grown in either the absence (cross-hatched bar) or presence (open bar) of 20 μ*M* TET for 5 days with either no added antioxidant or 24 hrs after the addition of 0.5 m*M* ascorbate or 0.1 m*M* α-tocopherol. Values are mean ± SEM for measurements from three separate cultures. TET and antioxidants significantly affected cytosolic cytochrome *c* ($P < 0.05$, ANOVA), but the TET × antioxidant interaction was not significant ($P > 0.05$, ANOVA). Bars labeled with an "a" indicate that mean cytosolic cytochrome *c* was higher in TET-treated cells exposed to α-tocopherol compared with TET-treated cells exposed to ascorbate or TET-treated cells not exposed to antioxidant ($P < 0.05$, Bonferroni contrasts).

localized in mitochondria and is released into the cytoplasm upon apoptotic stimulation (26). Once in the cytosol, pro-caspase-8 normally becomes activated through its interaction with the adapter molecules FADD/MORT1 after ligation of membrane-bound death receptors by tumor necrosis factor. Active caspase-8 then helps initiate the proteolytic cascade leading to apoptosis.

Our observation that TET-induced Cu deficiency caused a decrease in $\Delta\psi_m$ is consistent with a previous report of lower $\Delta\psi_m$ in heart mitochondria of Cu-deficient rats (27). However, even though $\Delta\psi_m$ was reduced in the Cu-deficient cells and it is known that apoptosis can be triggered by oxidative stress and that disruption of $\Delta\psi_m$ is an early event in the mitochondrial regulation of apoptosis, Cu deficiency alone did not activate caspase-8 or increase cytosolic cytochrome *c* content above basal levels in the TET-treated cells. This indicates that the level of oxidative stress caused by Cu deficiency in the TET-treated cells was insufficient to trigger apoptosis, even though $\Delta\psi_m$ was lowered. Also, exposure of HL-60 cells to ascorbate and α-tocopherol lowered $\Delta\psi_m$ independently of TET exposure, suggesting that these antioxidants at the concentrations used in our study can disrupt the mitochondrial electrochemical gradient. However, the decrease in $\Delta\psi_m$ occurred without a concurrent activation of caspase-8 or an increase in cytosolic cytochrome *c*, indicating that the disruption of $\Delta\psi_m$ by antioxidant treatment alone was insufficient to trigger apoptosis. Exposure of TET-treated cells to ascorbate or α-tocopherol increased caspase-8 activity, suggesting that the effects of Cu deficiency and antioxidants were additive and that their combined effects initiated apoptosis. In particular, α-tocopherol and TET treatment increased cytosolic cytochrome *c* above basal levels, suggesting that the combined effects of Cu deficiency and α-tocopherol triggered apoptosis through the mitochondrial pathway. Although caspase-8 is important for the transduction of extracellular apoptotic signals through membrane death receptors, our findings also suggest that internal apoptotic signals generated by overproduction of ROS by mitochondria in Cu-deficient cells are amplified by antioxidants, resulting in pro-caspase-8 activation. However, it is not clear whether caspase-8 activation results from the activation of existing cytosolic pro-caspase-8 or from release and subsequent activation of mitochondrial pro-caspase-8.

Although the induction of MnSOD indicates that cellular Cu deficiency increased oxidative stress in TET-treated cells, the failure of ascorbate and α-tocopherol to reverse the induction of MnSOD caused by TET treatment or to protect the growth and viability of TET-treated cells contraindicates protective, antioxidant roles for ascorbate and α-tocopherol in the TET-treated cells. Instead, our data suggest that ascorbate and α-tocopherol decrease the growth rate and the viability of HL-60 cells by promoting cell death when the cells are stressed as a result of Cu deficiency. This is consistent with other studies showing that Cu deficiency can promote apoptosis through mechanisms involving oxidative stress. For instance, it has been shown that oxidative injury is an important step in myocardial apoptosis during Cu deficiency in mice (28). It has also been shown that short-term Cu deficiency in neuroblastoma cells increases their susceptibility to apoptosis after oxidant challenge (29) and that prolonged Cu starvation in these cells causes mitochondrial damage and oxidative stress that lead to apoptosis (20).

It has been reported that millimolar concentrations of ascorbate can induce apoptosis in cultured cells through a mechanism involving oxidative stress and the formation of ascorbyl radical (30, 31). Although ascorbate did not induce cell death in HL-60 cells that were not treated with TET, it may have promoted cell death in TET-treated cells by adding to the level of oxidative stress already present in these cells as a result of Cu deficiency. Oxidative stress in the Cu-deficient cells may also have caused ascorbate to exhibit pro-oxidant activity through its interactions with transition metals in the cellular microenvironment. The presence of transition metals such as iron and Cu can cause ascorbate to act as a reducing agent and generate superoxide, hydrogen peroxide, and hydroxyl radical (32). Oxidative stress resulting from increased mitochondrial ROS production in TET-treated cells may promote the release of bound iron into intercellular compartments, where they can react with ascorbate to produce additional ROS, resulting in sufficient oxidative stress to trigger apoptosis. Although our study did not clearly define which apoptotic pathway is affected by ascorbate, the affected pathway does not appear to depend on the release of cytochrome *c* from

mitochondria because ascorbate did not elevate cytosolic cytochrome *c* above basal levels in Cu-deficient cells.

It has also been reported that α-tocopherol can promote cell death when the depletion of endogenous cellular antioxidants causes oxidation of α-tocopherol, resulting in the formation of the pro-oxidant, cytotoxic α-tocopheroxyl radical (33). In the present study, oxidative stress caused by Cu deficiency may have created an oxidative intercellular environment that promoted the oxidation of α-tocopherol and the formation of α-tocopheroxyl radical that triggered cell death in the TET-treated cells. In addition, α-tocopherol may also have stimulated cell death in Cu-deficient cells through a mechanism that does not directly involve oxidative stress and the formation of α-tocopheroxyl radical. It has been shown that α-tocopherol succinate activates various caspases, including caspase-8, and induces the translocation of cytochrome *c* to the cytosol in HL-60 cells (34). In our study, α-tocopherol acetate increased caspase-8 activity and elevated cytosolic cytochrome *c* above basal levels in Cu-deficient HL-60 cells. This suggests that α-tocopherol may have directly triggered apoptosis by promoting the release of cytochrome *c* and activation of caspase-8 in Cu-deficient HL-60 cells that were already susceptible to cell death because of perturbed mitochondrial membrane function and/or Cu deficiency.

Another potential mechanism through which ascorbate and α-tocopherol may increase caspase-8 activity in Cu-deficient HL-60 cells is related to the sensitivity of caspases to inactivation by oxidants. It has been shown that hydrogen peroxide has two effects on apoptosis in Jurkat T-lymphocytes, depending on its concentration (35). At low concentrations, hydrogen peroxide induces caspase activation and apoptosis. However, higher concentrations of hydrogen peroxide delay apoptosis by inhibiting caspase activity. The inhibition of caspases by hydrogen peroxide likely results from the oxidation of critical thiol residues. These findings suggest that cells need to maintain a reducing environment for optimal activation of caspases during apoptosis. Cu deficiency may alter the redox status of HL-60 cells and produce an oxidizing environment that is not conducive to caspase activation. Ascorbate and α-tocopherol may restore the cellular redox status and provide a sufficient reducing environment for caspase activation to occur in response to apoptotic signals generated in the Cu-deficient cells.

In conclusion, we found that producing Cu deficiency in HL-60 cells by treating them with the Cu chelator TET suppressed CCO activity and increased oxidative stress sufficiently to induce MnSOD. The level of oxidative stress was also sufficient to damage the inner mitochondrial membrane and lower the membrane potential. However, neither ascorbate nor α-tocopherol prevented oxidative damage to the mitochondria. Instead, ascorbate and α-tocopherol impaired growth and viability by promoting cell death of the Cu-deficient cells through reductions in mitochondrial membrane production and activation of caspase-8. Cell death caused by α-tocopherol did not depend on the release of cytochrome *c* from mitochondria, but cytochrome *c* release was a component of cell death caused by ascorbate. Although reductions in $\Delta\psi_m$, cytochrome *c* release, and caspase activation are early steps in apoptosis, our data are not sufficient to clearly indicate whether progression to cell death in the Cu-deficient cells exposed to ascorbate or α-tocopherol occurred through apoptosis or necrosis. However, the findings suggest that both ascorbate and α-tocopherol promote the death of cells experiencing oxidative stress caused by perturbations in the mitochondrial electron transport chain.

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