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ORIGINAL ARTICLE

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Ascorbate modulation of H_2O_2 and camptothecin-induced cell death in Jurkat cells

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Abstract The effect of ascorbate on cell death was examined in Jurkat cells (human T-cell leukemia) by incubation with dehydroascorbate (DHA), which is rapidly taken up by cells and efficiently reduced to ascorbate. Apoptosis was evaluated by caspase-3 activity in cell extracts and flow cytometry of annexin V-labeled cells. In parallel, necrosis was estimated by the release of lactate dehydrogenase. Minor effects on cell death were observed when Jurkat cells were incubated with either DHA alone (100–1,000 μM) or a single dose of 10 μM H_2O_2. However, pre-incubation with DHA followed by exposure to H_2O_2 clearly stimulated both apoptosis and necrosis. In complete contrast, pre-incubation of cells with DHA significantly inhibited apoptosis, but did not affect necrosis, induced by the topoisomerase I inhibitor camptothecin. Our results indicate that intracellular ascorbate can modulate cell death in a manner which depends upon the nature of the apoptotic stimulus, which in turn has critical implications regarding the mechanism and potential application of ascorbate in cancer therapy.

Keywords Dehydroascorbate · Reactive oxygen species · Topoisomerase inhibitors

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Abbreviations CPT: 20-S-camptothecin lactone · DHA: Dehydroascorbate · LDH: Lactate dehydrogenase · Ac-DEVD-AMC: Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin · PS: Phosphatidylserine · GSH: Glutathione · DTT: Dithiothreitol

Introduction

The utility of ascorbate (vitamin C) as an effective chemotherapeutic agent is highly controversial. In 1974, Cameron and Campbell reported catastrophic tumor hemorrhage and necrosis in a minority of terminally ill cancer patients treated with ascorbate [7]. Later on, results from double-blind clinical trials revealed no anticancer effects of ascorbate [16]. However, a critical difference between these latter two studies was the method of administration of ascorbate, i.e., intravenously vs orally, respectively. Oral administration leads to rapid saturation of ascorbate in plasma, whereas the intravenous method can yield much higher levels in cells and tissues [25].

A number of fundamental studies have underlined the potential benefit of high concentrations of ascorbate in cancer therapy. High concentrations of ascorbate have been reported to be toxic in several cancer cell lines [3, 18, 29, 30, 36] as well as in hollow fiber and ascitic liver tumor (TLT)-bearing mice [8, 39]. The combination of ascorbate and menadione appears to increase the life span of tumor-bearing mice [40]. In addition, cultured tumor cells [13, 37] and xenograph tumors of hematopoietic and epithelial cell lines in mice rapidly incorporate and accumulate ascorbate [1, 13, 37]. This accumulation is explained by initial oxidation of ascorbate near the tumor site followed by rapid incorporation of the oxidized form, dehydroascorbate (DHA) [1]. Interestingly, tumor cells accumulate DHA more than normal cells because the former generate more reactive oxygen species (ROS) and overexpress glucose transporters involved in DHA transport [38, 41, 42]. Much recent interest in ascorbate

stems from clinical studies showing that it potentiates the effects of arsenic trioxide in the treatment of promyelocytic leukemia [4, 17]. The purpose of the present work is to investigate the effects of ascorbate on the cytotoxic effects of H_20_2, which is a ubiquitous cellular oxidant implicated in the action of numerous therapeutic drugs, and camptothecin, which is a topoisomerase I inhibitor with application in cancer therapy.

ROS, such as superoxide radicals and H_20_2, play central roles in cell death pathways [9]. Either high acute doses (<1 mM) or low chronic doses (<2 μM/min) of H_20_2 can induce apoptosis of cancer cells in culture [2, 5]. The mechanism of cytotoxicity involves, among other processes, changes in energy metabolism, glutathione (GSH) depletion, and DNA damage [32, 33]. In addition, ROS are implicated in the induction of apoptosis by topoisomerase inhibitors. For example, the activation of NADPH oxidase and subsequent generation of H_20_2 is an important step in TAS-103 and beta lapachone-induced apoptosis in cultured cells [10, 24]. The effect of ascorbate on apoptosis induced by H_2O_2 and topoisomerase inhibitors is poorly understood.

Several studies have shown that ascorbate in combination with ROS enhances cell killing. For example, sodium 5,6-benzylidene-L-ascorbate or ascorbate decrease the survival of human squamous carcinoma and salivary gland tumor cells, and neuronal PC12 cells [3, 18, 29, 30, 36]. Ascorbate together with iron has been shown to enhance cyclosporine-induced peroxidation and killing of kidney epithelial cells [22]. Although ascorbate protected GSH deficient rabbit lens epithelium cells from H_20_2-induced killing, this effect decreased at higher concentrations of ascorbate, and even became toxic under certain conditions [34].

The most common method of incorporating ascorbate into cells, i.e., involving direct incubation with ascorbate, is problematic because the latter undergoes autooxidation in cell culture, which generates ROS in the medium [11]. The toxic effects of ascorbate in cell culture are probably mediated by ROS in agreement with increases in ascorbyl radical and H_2O_2-derived luminescence, and inhibition of killing by catalase [3]. For these reasons, we report the effect of ascorbate on H_2O_2-induced and camptothecin-induced cell death, using an alternative method to incorporate ascorbate into cells. This method involves incubation of cells with dehydroascorbate, the oxidized form of ascorbate, which is readily taken up by glucose transporters and efficiently reduced to ascorbate inside cells thus, circumventing possible generation of ROS in cell medium.

Materials and methods

Chemicals 20-S-camptothecin lactone (CPT) was purchased from Sigma Chemicals Co. (St Louis, MO) and hydrogen peroxide (H_20_2) was obtained from Fisher Scientific (Fair Lawn, NJ). The fluorogenic peptide derivative Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) was purchased from Calbiochem (La Jolla, CA) and annexin V-Alexa Fluor 488 conjugate from Molecular Probes, Inc. (Eugene, OR). All other chemicals were reagent grade and purchased from local sources.

Cell culture and treatments The human Jurkat cell line was obtained from the American Type Culture Collection (Manassas, VA). They were grown in suspension culture at 37°C under 5% CO_2 under a humidified atmosphere in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Exponentially growing cells were used throughout all experiments at a concentration of 2.5×10^5 cells/ml. Cells were treated with different concentrations of DHA in PBS for 30 min. Following incubation, cells were pelleted by centrifugation, and the supernatant was discarded and replaced with fresh medium with or without H_2O_2 (0–100 μM) or 1 μM CPT. The cells were subsequently incubated at 37°C for different periods of time.

Cell death analyses Cell lysates were prepared by suspending treated or untreated cells in lysis buffer containing 0.1% triton, 20% glycerol, 100 mM Hepes (pH 7.4), 5 mM DTT and 5 mM EDTA. The assay mixture consisted of 1 ml of the latter solution except without triton, 200 μg total protein from cell lysates and 20 μM Ac-DEVD-AMC. The assay mixture was incubated at 37°C for 1 h and the AMC liberated from the fluorogenic substrate was measured in a F-4500 fluorescence spectrophotometer (Hitachi) using 380 and 460 nm as the excitation and emission wavelengths, respectively. Caspase-3-like activity is expressed as relative fluorescence. The number of necrotic cells was estimated by the release of lactate dehydrogenase (LDH) activity into cell media according to the commercially available colorimetric assay (Roche Molecular Biochemicals, Mannhein, Germany). LDH release is expressed as a percentage: ((experimental value − low control value)/(high control value − low control value) × 100), where the low control value represents LDH activity in the cell media of untreated cells and the high control value is the total LDH activity, obtained by complete lysis of cells. Percentage LDH release is normalized to initial conditions, i.e., time zero or no DHA treatment. The initial percentage of LDH release varied from 25 to 40%. Cell death was also examined by flow cytometry. For these experiments, cells were labeled with annexin V-Alexa Fluor 488 conjugate according to the manufacturer's protocol (Molecular Probes, Eugene, OR), and they were analyzed by flow cytometry using a FACS Calibur cytometer (Becton Dickinson).

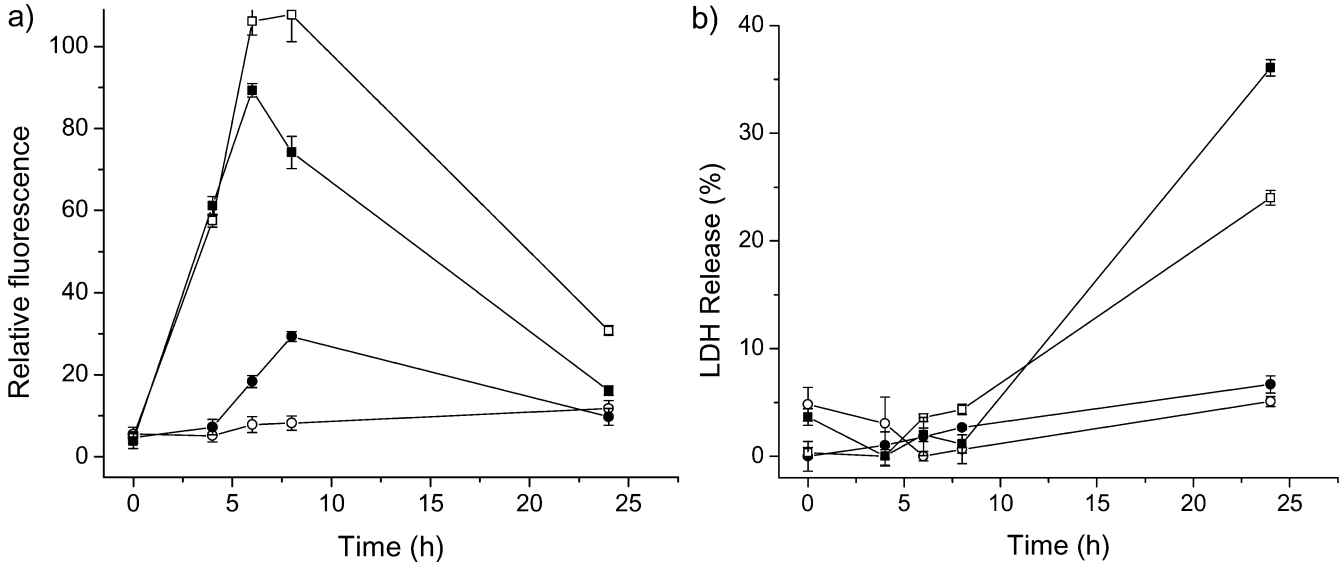
Ascorbate and glutathione analysis Intracellular ascorbate and glutathione were measured according to the method of Rose and Bode [28]. Ten million cells were washed three times with ice-cold chelex treated PBS, suspended in 200 mM phosphate buffer pH 3.0 containing 0.1 mM EDTA, and subjected to three freeze-thaw cycles (4 to −80°C). Ascorbate and glutathione were quantified by HPLC. The HPLC system consisted of a LC-10AD pump (Shimadzu, Kyoto), SIL-HT autoinjector (Shimadzu) and an electrochemical detector (ESA, Inc., Chelmsford, MA) equipped with a 5010 flow cell. The analysis was carried out using an octadecylsilyl Inertsil 5 μm 150×4.6 mm internal diameter column (CSC, Montreal) with 200 mM phosphate, pH 3.0, as the mobile phase. Ascorbate and glutathione were detected by their oxidation at 300 and 600 mV, respectively, vs Pd reference electrode. The amounts of ascorbate and glutathione were normalized to total protein as determined by the Bradford assay (Bio-Rad) and their concentration within cells was calculated assuming an average amount of protein of 0.025 mg/10^6 cells and volume of 200 nl/10^6 [23].

Results

Jurkat cells were incubated with the oxidized form of ascorbate DHA rather than with the reduced form in order to preclude ROS generation in cell medium. The accumulation of ascorbate in cells increased linearly with the concentration of DHA and reached saturation in the range of 200–500 μM DHA. A concentration of DHA of 1 mM was chosen to assure that the accumulation of ascorbate was constant in all experiments. Upon incubation with 1 mM DHA, the concentration of ascorbate in cells reached 22 mM. This concentration decreased to 15 mM after 2 h and to 4 mM after 6 h of incubation in cell culture. In comparison, the concentration of glutathione in cells was 4 mM and it did not change for the next 6 h of incubation in cell culture. To evaluate the effect of ascorbate on H_20_2-induced cell death, cells were first incubated with DHA to elevate intracellular ascorbate, washed twice to eliminate DHA decomposition products, and then exposed to H_20_2. The proportion of cells undergoing apoptosis was estimated by cleavage of Ac-DEVD-AMC, a specific substrate of caspase-3 in whole cell extracts. In parallel, cell death by necrosis was determined by the release of LDH, a cytoplasmic enzyme that rapidly accumulates in cell culture medium upon damage of the plasma membrane.

Induction of caspase-3-like activity in Jurkat cells was detected upon exposure to 25 μM H_2O_2 but not to 10 μM H_2O_2. Higher concentrations of H_2O_2 (> 25 μM) gave lower caspase-3-like activity and greater LDH release. Similarly, Hampton and Orrenius [15] reported a decrease in caspase-3-like activity in Jurkat cells exposed to high concentrations of H_2O_2 (> 50 μM). The effect of DHA on H_2O_2-induced caspase-3-like activity depended on the concentration of H_2O_2. At the low concentration of H_2O_2 (10 μM), DHA significantly increased caspase-3-like activity from 7.8 ± 0.5 to 18.4 ± 0.8 at 6 h and from 8.2 ± 0.4 to 29.3 ± 0.5 at 8 h (Fig. 1). In contrast,

Fig. 1 Time-course of H_20_2-induced apoptosis. Jurkat cells were exposed to H_20_2 and incubated in fresh RPMI medium at 37°C for various time (0, 4, 6, 8, and 24 h). Relative fluorescence, i.e., **a** caspase-3-like activity and **b** LDH release were determined as described in the "Materials and methods." LDH release was normalized to cells at time zero. Data points ($n = 3$; error bars; ± SD): 10 μM H_20_2 (*open circle*); 1 mM DHA followed by 10 μM H_20_2 (*filled circle*); 25 μM H_20_2 (*open square*) and 1 mM DHA followed by 25 μM H_20_2 (*filled square*). The differences between caspase-3-like activity at 6 and 8 h were significant for all conditions ($P < 0.05$, *t*-test). LDH release at 24 h was significantly higher for 25 μM H_20_2 plus DHA compared to 25 μM H_20_2 alone, which were both higher than values for 10 μM H_20_2 ($P < 0.05$, *t*-test)



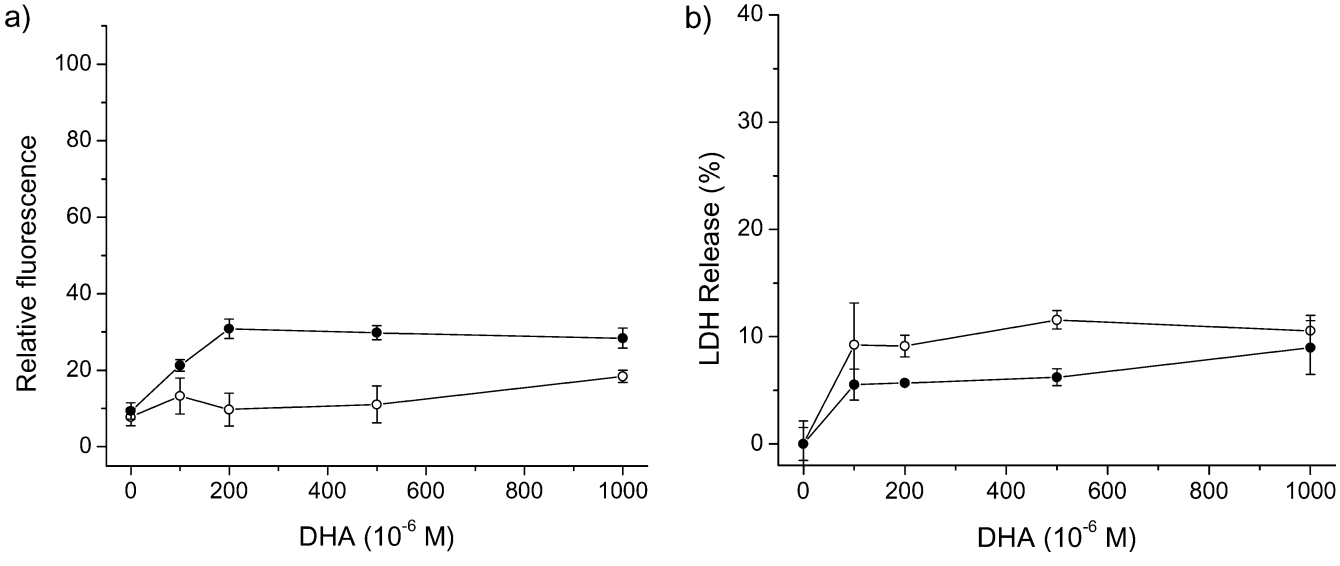
the high concentration H_2O_2 (25 μM), resulted in a decrease of caspase-3-like activity (Fig. 1). Both of these effects can probably be attributed to a sensitization of cell death by intracellular ascorbate. The apparent reduction in caspase-3-like activity at 25 μM can be explained by a shift of apoptotic cells towards necrotic cell death, rather than to an inhibition of apoptosis [12]. Indeed, the extent of LDH release after 24 h in cell culture was 24% for cells exposed to 25 μM H_20_2 alone compared 36% for cells treated with DHA and then exposed to H_20_2 (Fig. 1b).

The effect of DHA concentration on caspase-3-like activity and LDH release was examined at 10 μM H_20_2 (Fig. 2). H_20_2-induced caspase-3-like activity reached a maximum following treatment with 200 μM DHA. There was no significant change in this activity at higher concentrations of DHA. The concentration of intracellular ascorbate following treatment with 200 μM DHA was 14 mM, suggesting that a lower concentration than that obtained following treatment with 1 mM DHA (22 mM) is sufficient to sensitize cells to H_2O_2-induced cell death.

The effect of DHA on cell death induced by H_20_2 was examined by flow cytometry using annexin V-Alexa Fluor 488 conjugate and propidium iodide as fluorescent probes. Annexin V identifies apoptotic cells by binding to phosphatidylserine, which translocates from the inner to the outer leaflet of the plasma membrane as an early event in leukocyte apoptosis [20]. Propidium iodide is a fluorescence probe that diffuses into cells as a result of plasma membrane damage, indicative of necrosis. The percentage of cells exhibiting annexin V fluorescence was less than 2% in either control or DHA treated cells. For cells that were not treated with DHA but exposed to 10 or 25 μM H_2O_2, there was a significant increase in the percentage of cells exhibiting annexin V fluorescence indicative of apoptosis and necrosis (Fig. 3a,c). In contrast, pre-treatment of cells with DHA followed by exposure to H_2O_2 (10 or 25 μM) led to a shift toward early apoptotic cells, and late apoptotic and/or necrotic cells, relative to the situation for cells not treated with DHA (Fig. 3). Because there was no significant increase of LDH release at 6 h (Fig. 2b), cells that tested positive for propidium iodide and annexin V binding (upper right quadrant; Fig. 3) are more likely to be in late apoptosis than in necrosis. Combining the results from early and late apoptosis, the percentage of apoptosis appears to increase from 18.7 to 30.2% for 10 μM H_2O_2 and from 16.3 to 40.9% for 25 μM H_2O_2 upon pre-treatment with DHA (Fig. 3a,b, 10 μM H_2O_2; Fig. 3c,d, 25 μM H_2O_2). This is consistent with the analysis of caspase-3-like activity, showing that DHA also sensitizes cells to killing by apoptosis (Figs. 1, 2). On the basis of caspase-3-like activity and annexin V binding, we conclude that DHA sensitizes Jurkat cells to H_20_2-induced apoptosis. Interestingly, DHA sensitized annexin V binding while it decreased the level of caspase-3-like activity at the higher concentration of H_2O_2. This may reflect differences in timing of the two events in apoptosis such that phosphatidylserine translocation is an early event while caspase-3 induction is a later downstream event. An alternative is that H_2O_2 inhibited caspase-3-like activity at 25 μM, although this is doubtful because H_2O_2 disappears in 2 h whereas caspase-3-like activity slowly increases and attains a maximum at 6–8 h [15, 21].

Camptothecin (CPT) is a topoisomerase I inhibitor that efficiently induces apoptosis in Jurkat cells. DHA strongly reduced caspase-3-like activity associated with CPT-induced apoptosis but did not have a significant

Fig. 2 Effect of DHA on H_20_2-induced cell death. Jurkat cells were treated with DHA in PBS for 30 min, pelleted by centrifugation, and exposed to H_20_2 in fresh RPMI medium at 37°C. **a** Caspase-3-like activity and **b** LDH activity were determined after 6 h incubation. Data points ($n = 3$; error bars; ±SD): control (*open circle*) and 10 μM H_20_2 (*filled circle*). The differences of caspase-3-like activity between control and 10 μM H_20_2 were significant at 200, 500 and 1,000 μM DHA ($P < 0.05$, *t*-test)



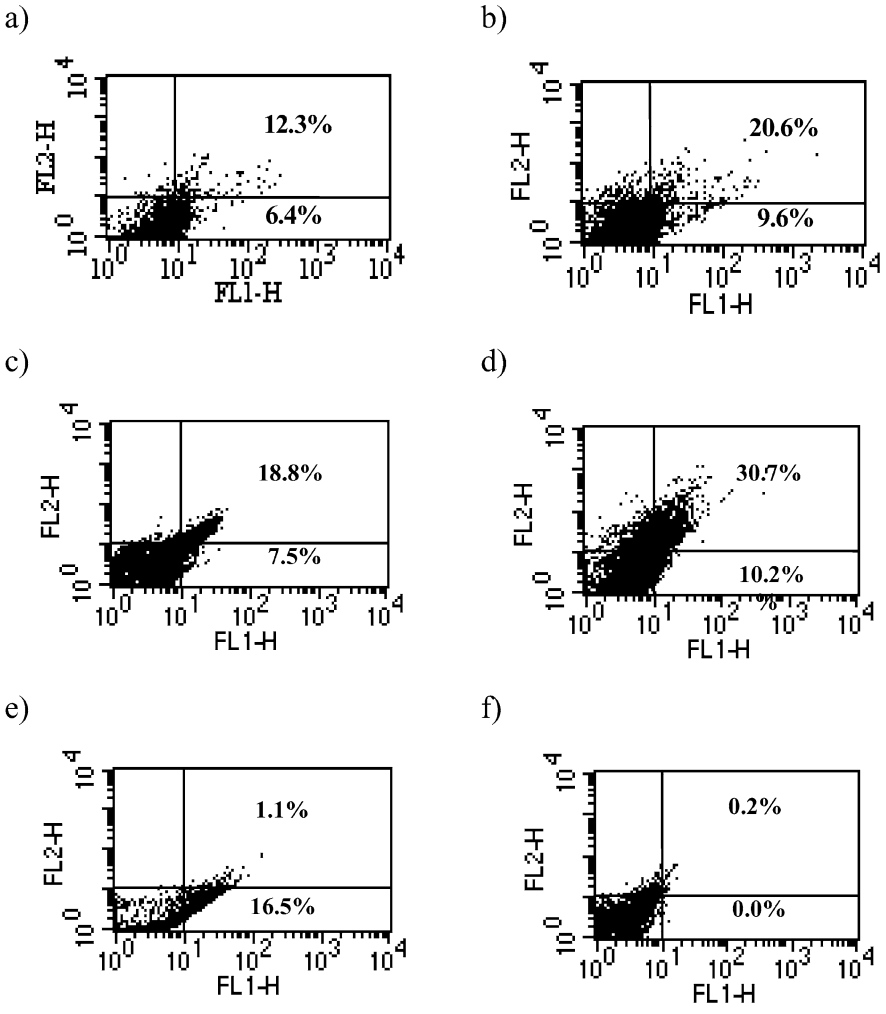


Fig. 3 Analysis of cell death by flow cytometry. Cells were fixed with annexin V-Alexa Fluor 488 and propidium iodide for flow cytometric analyses. FL1-H (*x*-axis) refers to annexin V-AlexaFluor fluorescence and FL2-H (*y*-axis) refers to propidium iodide fluorescence. *Lower left quadrant*, viable cells; *lower right quadrant*, early apoptotic cells and *upper right quadrant*, necrotic and/or late apoptotic cells. Treatment with DHA was carried out in PBS and the cells were exposed to H_20_2 or CPT in fresh RPMI medium. Cells were then incubated at 37°C for 6 h before analyses. **a** no DHA and 10 μM H_20_2; **b** 1 mM DHA and 10 μM H_20_2; **c** no DHA and 25 μM H_20_2; **d** 1 mM DHA and 25 μM H_20_2; **e** no DHA and 1 μM CPT; **f** 1 mM DHA and 1 μM CPT. Each experiment was repeated 2–3 times. The percentage of cells in the *upper right quadrant* (necrotic or late apoptotic) was significantly different between **a** and **b**, and between **c** and **d** ($P < 0.05$, *t*-test). The percentage of cells in the *lower right quadrant* (early apoptotic) was significantly different between **e** and **f** ($P < 0.05$, *t*-test)

effect on necrosis (Fig. 4). Again, this effect appears to become saturated at about 200 μM DHA. We also observed an inhibition of apoptosis using annexin V labeling and flow cytometry. The percentage of early apoptotic cells induced by 1 μM CPT was significantly reduced when the cells were pre-treated with DHA (Fig. 3e,f). These results cannot be explained by changes in cell cycle induced by DHA because there was no change in either the doubling time of cells or in the distribution of cells by flow cytometry (data not shown).

Discussion

The use of DHA as a means to examine the effects of intracellular ascorbate has been limited to only a few studies. Previously, Puskas et al. [26] reported that short-term incubation with DHA (400 μM; 20 min to 2 h) decreased cell survival whereas long-term exposure (36 h) increased cell survival of Jurkat cells exposed to 100 μM H_2O_2. These apparently opposing effects can be attributed to the ability of intracellular ascorbate to increase GSH in the long-term and curtail potentially toxic effects of elevated ascorbate. Similar results have been reported for other cell lines [6, 34]. For example, Bijur et al. [6] reported that DHA (50 μM for 2 h) greatly reduced the survival of Chinese hamster ovary cell line AS52 but only when they were depleted of GSH before exposure to ROS by xanthine oxidase and hypoxanthine. Thus, the ability of ascorbate to sensitizes H_2O_2-induced cell death depends on the concentration of intracellular GSH.

The ability of ascorbate to induce apoptosis as observed here in Jurkat cells sharply contrasts recent studies with HL-60 cells where high levels of intracellular ascorbate protected against H_2O_2-induced cell death [12]. The reason for this differential response to H_2O_2-induced cell death is not clear, but can likely be attributed to differences in the intracellular concentration of GSH. Indeed, the concentration of GSH in Jurkat cells was 4 mM GSH in our experiments, whereas that in HL-60 was 12.7 mM [14]. On the other hand, we did not observe a change in GSH during the first 6 h following ascorbate treatment, suggesting that Jurkat cells rapidly regenerate GSH required for the reduction of DHA.

DNA topoisomerase I and II inhibitors induce apoptosis in various cell lines [19, 31] and this is due to DNA-protein complex formation stabilized by DNA

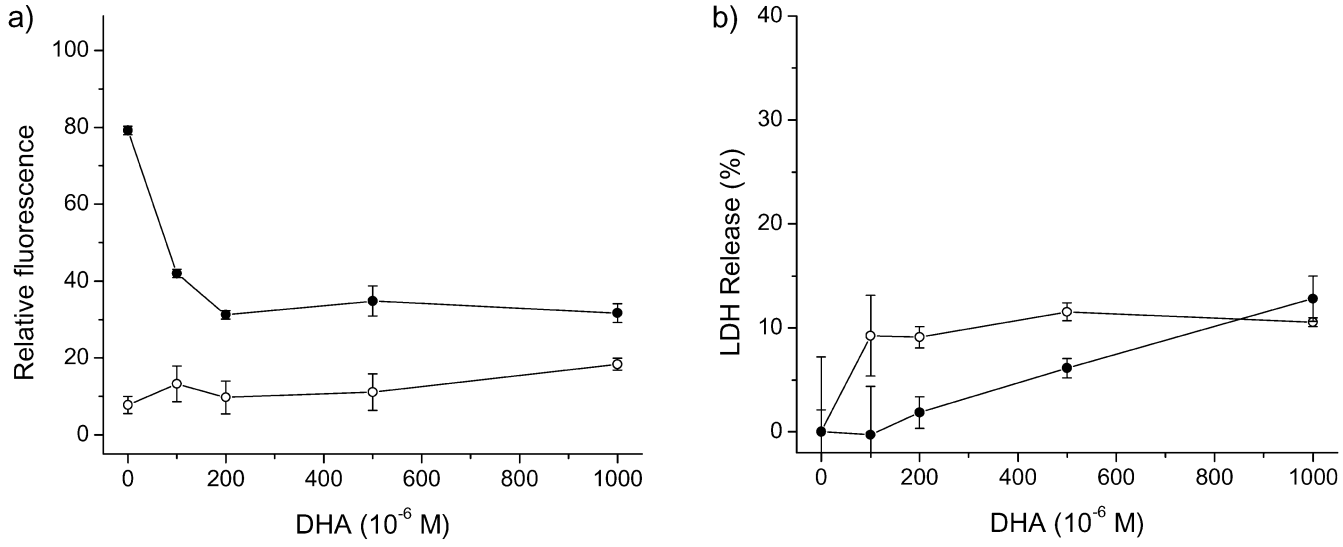


Fig. 4 Effect of DHA on CPT-induced cell death. Jurkat cells were treated with different concentrations of DHA in PBS for 30 min. They were then pelleted by centrifugation and treated with 1 μM CPT in fresh RPMI medium at 37°C for 6 h. Following incubation period, **a** caspase-3-like activity and **b** LDH release were assessed as in Fig. 1. Data points ($n=3$; error bars; ±SD): control (*open circle*); 1 μM CPT (*filled circle*). The differences in caspase-3-like activity between cells exposed to CPT and control cells were significant at all concentrations of DHA ($P<0.05$, *t*-test)

topoisomerase I inhibitors that ultimately signal the onset of apoptosis [19, 31]. Our results show that DHA-loading inhibits CPT-induced apoptosis (Figs. 3, 4), suggesting that ROS generation is an early event in CPT-induced apoptosis. Whereas it is generally accepted that ROS are involved in the action of some topoisomerase II inhibitors, such as beta-lapachone [10], no influence of antioxidants on topoisomerase I inhibitors, such as CPT, has been previously reported. Ascorbate does not interfere with the activation of c-Jun NH2-terminal kinase (JNK) or caspase-3-like activity induced by CPT and *N*-acetylcysteine administration fails to block CPT-induced apoptosis in HL-60 cells [35]. On the other hand, caspase-3 has been implicated in the generation of ROS by its ability to gain access to the mitochondrial intermembrane space and disrupt complex I and complex II of the electron transport chain [27]; ROS generated in this way appear to amplify the apoptotic signal. Using digitonin-permeabilized Jurkat cells, the authors showed that the generation of ROS by the associated disruption in electron transport is inhibited by the caspase inhibitor, zVAD-fmk.

The present study demonstrates that high concentrations of intracellular ascorbate sensitize Jurkat cells to H_2O_2-induced apoptosis and necrosis. This suggests that ascorbate can sensitize cancer cells to therapeutic agents that generate H_2O_2 or that compromise antioxidant defenses against ROS. For instance, the potentiating effects of ascorbate toward vitamin K3-induced and arsenic trioxide-induced cell killing may be explained by a change in cellular redox status as a result of increased ROS generation or reduced intracellular GSH. On the other hand, we show that ascorbate diminishes the ability of topoisomerase I inhibitors to induce apoptosis. This suggests that the presence of high concentrations of ascorbate can limit the effectiveness of topoisomerase I inhibitors in cancer therapy. Further research will be necessary to establish the utility of high concentrations of ascorbate in cancer therapy alone and in combination with other drugs.

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