α-lipoic acid induces apoptosis in human colon cancer cells by increasing mitochondrial respiration with a concomitant $O_2^-$-generation

U. Wenzel, A. Nickel and H. Daniel
Molecular Nutrition Unit, Department of Food and Nutrition, Technical University of Munich, Hochfeldweg 2, D-85350 Freising, FRG

The antioxidant α-lipoic acid (ALA) has been shown to affect a variety of biological processes associated with oxidative stress including cancer. We determined in HT-29 human colon cancer cells whether ALA is able to affect apoptosis, as an important parameter disregulated in tumour development. Exposure of cells to ALA or its reduced form dihydrolipoic acid (DHLA) for 24 h dose-dependently increased caspase-3-like activity and was associated with DNA-fragmentation. DHLA but not ALA was able to scavenge cytosolic $O_2^-$ in HT-29 cells whereas both compounds increased $O_2^-$-generation inside mitochondria. Increased mitochondrial $O_2^-$-production was preceded by an increased influx of lactate or pyruvate into mitochondria and resulted in the down-regulation of the anti-apoptotic protein bcl-XL. Mitochondrial $O_2^-$-generation and apoptosis induced by ALA and DHLA could be prevented by the $O_2^-$-scavenger benzoquinone. Moreover, when the lactate/pyruvate transporter was inhibited by 5-nitro-2-(3-phenylpropylamino) benzoate, ALA- and DHLA-induced mitochondrial ROS-production and apoptosis were blocked. In contrast to HT-29 cells, no apoptosis was observed in non-transformed human colonocytes in response to ALA or DHLA addition. In conclusion, our study provides evidence that ALA and DHLA can effectively induce apoptosis in human colon cancer cells by a prooxidant mechanism that is initiated by an increased uptake of oxidizable substrates into mitochondria.

Keywords: HT-29 human colon cancer cells; mitochondrial apoptosis pathway; monocarboxylate transporter; superoxide anion generation.

Abbreviations: Ac-DEVD-AMC, acetyl-aspartyl-glutamylvalyl-aspartyl-amino-4-methyl-coumarine; ALA, Alpha-lipoic acid; CHAPS, 3-[[(cholamidopropyl)-dimethyl-ammonium]1-propane-sulfonate; CLSM, Confocal laser scanning microscopy; DTT, dithiotreitol; GAP-DH, glyceraldehyde-3-phosphate dehydrogenase; ModEM, modified Eagle medium; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoate; PDH, pyruvate-dehydrogenase; proxyl fluorescamine.


Introduction

Oxidative stress is suggested to play a causative role in different degenerative diseases such as cancer. Dietary or endogenous antioxidants do interfere with these processes and amongst those alpha-lipoic acid (ALA) has recently gained considerable attention. ALA has been reported to have beneficial effects in patients with advanced cancers by increasing the glutathione peroxidase activity and by reducing oxidative stress. On a molecular basis ALA and DHLA (dihydrolipoic acid) were shown to alter the expression of the protooncogene c-fos when induced by 12-O-tetradecanoyl-phorbol-13 acetate (TPA) and TPA effects were found to be associated with the production of $O_2^-$- whereas DHLA reduced the expression of c-fos, ALA increased expression and it was suggested that the suppression of c-fos expression is due to the capability of DHLA but not of ALA to scavenge $O_2^-$.

Apoptosis is another mechanism in which the presence of reactive oxygen species (ROS) plays an important role and apoptosis is generally impaired in cancer cells. We previously showed in HT-29 colon cancer cells that apoptosis induction by flavone is associated with a high rate of mitochondrial $O_2^-$ production and that scavenging mitochondrial ROS prevents apoptosis to occur. That apoptosis induction by flavone is specific for tumor cells was demonstrated by a lack of flavone effects in non-transformed colonocytes which could be explained by a higher antioxidant status of these cells. Similar findings were obtained with ALA. It was shown to trigger apoptosis in human cancer cell lines while inducing a reversible cell-cycle arrest but failed to induce apoptosis in non-transformed cell lines. Moreover, ALA could potentiate apoptosis in human leukemia cells and based on its antioxidant properties it was suggested that ALA may...
promote a reducing environment that is required for the activation of caspases.\textsuperscript{17}

Whereas ALA in leukemia cells predominantly showed proapoptotic effects, in neurons\textsuperscript{18} and in hepatocytes\textsuperscript{19} ALA appears to protect cells from apoptosis by its antioxidative properties. So, depending on the cellular background, ALA possesses quite different biological activities in tumor cells and non-transformed cells. Since we demonstrated that scavenging of mitochondrial \( \text{O}_2^- \) by antioxidants such as ascorbic acid or benzoquinone blocks drug-induced caspase-activation and apoptosis induction in colon cancer cells,\textsuperscript{20} we investigated whether ALA and its reduced form DHLA can similarly affect apoptosis in HT-29 colon cancer cells. Caspase activation and nuclear fragmentation served as early and late apoptosis markers, respectively. Changes in the level of the apoptosis relevant bcl-x\textsubscript{L} protein were determined by Western-blotting and confocal microscopy was used to determine the role of \( \text{O}_2^- \) in the apoptotic response of the cells. To assess whether apoptosis execution by ALA or DHLA is specific for colon tumor cells, the non-transformed human colonocyte cell line NCOL-1\textsuperscript{21} served as a control.

Materials and methods

Cell culture

HT-29 cells (passage 106) were provided by American Type Culture Collection and used between passage 150 and 200. HT-29 cells were cultured and passaged in RPMI-1640 supplemented with 10% FCS and 2 mM glutamine. Antibiotics added to the media were 100 U/ml penicillin and 100 \( \mu \)g/ml streptomycin. NCOL-1 cells (passage 50) were a kind gift of Prof. Clifford W. Deveney and Dr. Michael J. Rutten, School of Medicine, Oregon Health Sciences University, Oregon, USA. Cells were cultured and passaged in DMEM/Hepe/glutamine supplemented with 10% FCS, MEM amino acids, BME vitamin solution and 1 mM human recombinant epidermal growth factor. Antibiotics added to the media were 200 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, 12.5 \( \mu \)g/ml gentamicin and 1 \( \mu \)g/ml fungizone. All cultures were maintained in a humidified atmosphere of 95% air and 5% \( \text{CO}_2 \) at 37°C. Cells were passaged at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA (all materials for cell culture were from Invitrogen, Karlsruhe, Germany).

Detection of apoptosis

Caspase-3-like activity was measured as described previously,\textsuperscript{13} based on the method of Nicholson \textit{et al.}\textsuperscript{22} In brief, HT-29 cells were seeded at a density of \( 5 \times 10^5 \) per well onto 6-well plates (Renner, Dannstadt, Germany) and allowed to adhere for 24 h. Cells were then exposed for the times indicated to the test compounds. Subsequently, cells were trypsinized, cell numbers were determined and then the cells were centrifuged at 2500 g for 10 min. Cytosolic extracts were prepared by adding 750 \( \mu \)l of a buffer containing 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 10 \( \mu \)g/ml pepstatin A, 20 \( \mu \)g/ml leupeptin, 10 \( \mu \)g/ml aprotinin and 10 mM HEPES/KOH, pH 7.4, to each pellet and homogenizing by 10 strokes. The homogenate was centrifuged at 100,000 \( \times \) g at 4°C for 30 min and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC (Calbiochem, Bad Soden, Germany) at a final concentration of 20 \( \mu M \). Cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using a fluorescence microtiter plate reader (Fluoroskan Ascent, Labsystems). Nuclear fragmentation as a late marker of apoptosis was determined by staining of DNA with Hoechst 33258 (Sigma, Deisenhofen; Germany). HT-29 cells (3 \( \times \) \( 10^4 \)) were grown on glass slides placed into Quadriperm wells (Merck, Darmstadt, Germany) and then incubated with the test compounds for 24 h. Thereafter, cells were washed with PBS, allowed to air-dry for 30 min and then fixed with 2% paraformaldehyde prior to staining with 1 \( \mu \)g/ml Hoechst 33258 and visualization under an inverted fluorescence microscope (Leica DMIRBE). Photographs were made from at least three independent cell batches and apoptotic cells were determined according to the number of cells displaying chromatin condensation and nuclear fragmentation versus total cell counts.

\( \text{O}_2^- \)-detection

For detection of \( \text{O}_2^- \) inside HT-29 colonocytes the cells were loaded with 50 \( \mu M \) proxylfluorescamine (Bioprobes, Leiden, The Netherlands) for 2 h. 200 \( \mu M \) cysteine were added to the incubation media to yield an increase in the emission of proxylfluorescamine fluorescence due to the reduction of the fluorophore nitroxide to its corresponding hydroxylamine in the presence of superoxide.\textsuperscript{23} Subsequently to the loading with fluorophore cells were washed free of medium with a modified Krebs-buffer, containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl\textsubscript{2}, 1 mM MgSO\textsubscript{4}, 0.3 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.3 mM KH\textsubscript{2}PO\textsubscript{4}, 10 mM glucose, 200 \( \mu M \) cysteine and 10 mM Heps/Tris, pH 7.4. \( \text{O}_2^- \)-generation inside HT-29 cells was achieved by the addition of 50 \( \mu M \) camptothecin. The amount of \( \text{O}_2^- \) in HT-29 cells was followed over 3 h by measuring the fluorescence of proxylfluorescamine at 460 nm after excitation at 390 nm using the fluorescence microtiter plate reader.

\( \text{O}_2^- \) in mitochondria of HT-29 cells were visualized by confocal laser scanning microscopy (CLSM) using a TCS