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Dihydro-alpha-lipoic acid has more potent cytotoxicity than alpha-lipoic acid

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Abstract Alpha-lipoic acid has been shown to possess cancer-cell-killing activity via activation of the apoptosis pathway. In this study, the cytotoxic activities of alpha-lipoic and dihydro-alpha-lipoic acid were compared in HL-60 cells. The cell-killing activity of dihydro-alpha-lipoic acid was higher than that of alpha-lipoic acid. Both alpha-lipoic and dihydro-alpha-lipoic acid induced caspase-3 cleavage and internucleosomal DNA fragmentation in treated cells. On the other hand, apparent necrotic or late-stage apoptotic cell populations could be detected in dihydro-alpha-lipoic acid cells but not in those treated with alpha-lipoic acid. Moreover, dihydro-alpha-lipoic acid, but not alpha-lipoic acid, induced marked mitochondrial permeability transition. Antioxidants could not prevent dihydro-alpha-lipoic- or alpha-lipoic-acid-induced cell death. In addition, dihydro-alpha-lipoic and alpha-lipoic acid did not up-regulate cellular reactive oxygen level. These results indicated that dihydro-alpha-lipoic acid exerts more potent cytotoxicity than alpha-lipoic acid through different cytotoxic actions.

Keywords Lipoic acid · Dihydro lipoic acid · Apoptosis · Necrosis · Caspase

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Introduction

Alpha-lipoic acid (ALA), 6,8-dithiooctanoic acid, is a naturally occurring antioxidative compound and is an essential cofactor in humans, functioning as a coenzyme in various biological processes. ALA is present as lipoyllysine in vegetables and animal tissues (Lode et al. 1997). Due to its potent antioxidant activity in vitro and in vivo, it is utilized as a preventive agent in diabetes mellitus, hypertension, and hepatic disorders (Bustamante et al. 1998; Vasdev et al. 2000; Packer et al. 2001). Recent studies revealed that ALA induces cell cycle arrest in non-transformed cells and apoptotic cell death in several cancer cells (Marsh et al. 2005; Wenzel et al. 2005; Moungjaroen et al. 2006). ALA is reduced to dihydro-ALA (DHLA) via mitochondrial dihydrolipoamide dehydrogenase, cytosolic glutathione reductase, and thioredoxin reductase (Slepneva et al. 1995; Arner et al. 1996; Bilska and Wlodek 2005). Therefore, it is important to determine which is the active compound, ALA or DHLA, to exert various beneficial effects. It is known that ALA and DHLA do not show equivalent efficacy in several physiological activities (Muller and Menzel 1990; Lapenna et al. 2003; Coleman et al. 2006). The present study was performed to elucidate the differences in the cytotoxic activities of ALA and DHLA.

Materials and Methods

Materials. ALA, *N*-acetyl-L-cysteine (NAC) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and anti-β-actin clone AC-15 were purchased from Sigma (St. Louis, MO). DHLA was obtained from Calbiochem (San Diego, CA). (±)-Alpha-tocopherol (Toc) was purchased from Wako Pure Chemicals (Osaka, Japan). Peroxidase-conjugated anti-mouse IgG and peroxidase-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies for cleaved caspase-3, which recognized large fragments of 17 and 19 kDa, were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture. HL-60 cells were purchased from RIKEN Bioresource Center (Tsukuba, Japan). Cells were maintained in RPMI1640 medium (Sigma) supplemented with 10% fetal bovine serum containing 100 units/ml penicillin G and 100 μg/ml streptomycin. Cells were subcultured twice a week. For experiments, the cell number was adjusted to 1.0×10^5 cells/ml.

Internucleosomal DNA fragmentation. Internucleosomal DNA fragmentation was evaluated using agarose gel electrophoresis. Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 10 mM Tris–HCl (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. Cell lysate was treated with 100 μg/ml RNase A (Sigma) at 37°C for 1 h followed by 100 μg/ml proteinase K (Sigma). Thereafter, DNA was precipitated in 0.5 M NaCl in 50% isopropanol and dissolved in Tris-EDTA buffer. DNA samples were subjected to 2% agarose gel electrophoresis and detected after SYBR green dye staining.

Western blotting analysis. Cleaved caspase-3 was detected by Western blotting analysis. At the end of the culture period, cells were lysed in 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, 2% Triton X-100, 2 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, and 1/50 vol. protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Protein concentrations were measured using the BCA protein assay reagent (Pierce, Rockford, IL). Lysates containing 10 μg of protein were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels, transferred onto polyvinylidene difluoride Hybond-P membranes (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Blocking was performed using 3% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TTBS), and antibodies were diluted in Can Get Signal solutions 1 and 2 (Toyobo, Tokyo, Japan). The membranes were washed with TTBS after each antibody-binding reaction. Detection of each protein was performed using an ECL Plus kit (Amersham Pharmacia).

Annexin-V staining. At the end of the culture period, cells were washed with ice-cold PBS and stained using a commercial kit (BenderMedsystems, Vienna, Austria). Experiments were performed in accordance with the manufacturer's protocol. After staining, cells were subjected to flow cytometric analysis (EPICS XL; Beckman Coulter, Fullerton, CA).

Mitochondrial permeability transition. At the end of the culture period, cells were washed with ice-cold PBS and stained with 120 μM MitoTracker Orange for 45 min at 37°C. After staining, cells were subjected to flow cytometric analysis (EPICS XL).

Intracellular redox state. At the end of the culture period, cells were washed with ice-cold PBS and stained with 50 μM DCFH-DA, which is hydrolyzed and oxidized to the fluorescent substance DCF, for 30 min at 37°C. After staining, cells were subjected to flow cytometric analysis (EPICS XL).

Statistical analysis. Data were analyzed by Student's *t* test to evaluate the significance of differences at $P<0.05$. Flow cytometric data shown in Table 1 were analyzed using the Tukey–Kramer test.

Results

First, the cytotoxic activities of ALA and DHLA on HL-60 cells were compared. HL-60 cells were treated with 0.5 and 1 mM of ALA or DHLA for 24 h, and viable cell numbers were counted. The results showed that ALA slightly reduced viable cell number at 0.5 and 1.0 mM (Fig. 1). On the other hand, DHLA exerted significant cytotoxicity at 0.5 mM, and viable cell number in 1 mM DHLA was reduced to 7% of that in untreated cells.

Next, the mechanisms of ALA- and DHLA-induced cell death in HL-60 cells were investigated. Several previous

Table 1. Flowcytometric analysis for Annexin-V and PI staining after ALA or DHLA treatment in HL-60 cells

Population(%)	Early apoptosis	Terminal apoptosis or necrosis
None	2.9 ± 0.6^{a}	2.1 ± 0.4^{a}
ALA	28.7 ± 1.3^{b}	3.6 ± 0.6^{a}
DHLA	15.8 ± 3.2^{ab}	8.0 ± 1.3^{b}

Data are means±SE of three independent experiments. Values without containing any common *superscript letter* are significantly different each other at $P<0.05$. Cells were harvested at 1.0×10^5 cells/ml and cultured for 24 h with or without ALA (1.0 mM) or DHLA (0.5 mM). At the end of the culture period, cells were stained with FITC-conjugated Annexin-V and propidium iodide using commercial kit. After staining, cells were subjected to flowcytometric analysis.

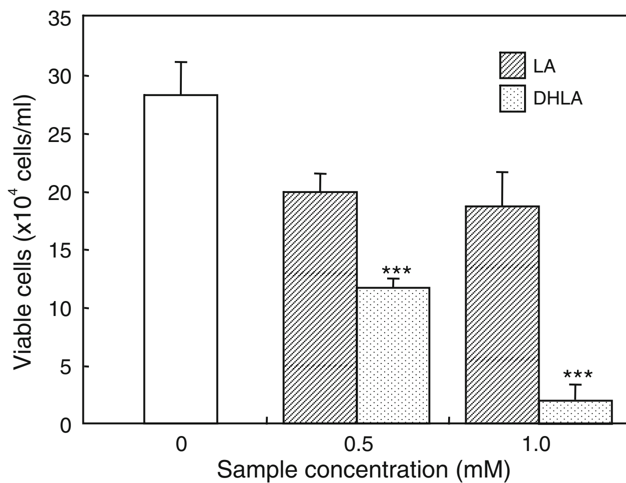

Figure 1. Comparison of the cytotoxic activity between ALA and DHLA in HL-60 cells. Data are means±SE for three samples. Significant differences from control value are indicated by *asterisks*: *$P<0.05$, **$P<0.01$, ***$P<0.001$. Cells were harvested at 1.0×10^5 cells/ml and cultured for 24 h with or without various concentrations of ALA or DHLA. Viable cells were counted using the trypan blue exclusion method.

studies indicated that ALA induced apoptotic cell death in various cell lines. Therefore, whether DHLA could induce apoptosis in HL-60 cells was examined. As shown in Fig. 2, ALA induced internucleosomal fragmentation of DNA. In addition, internucleosomal DNA fragmentation was also recognized in DHLA-treated cells. On the other hand, the degree of internucleosomal fragmentation of DNA was similar in ALA- and DHLA-treated cells despite differences in their cytotoxic activities. Cleaved caspase-3 was also detected in both ALA- and DHLA-treated cells at 12 and 24 h of incubation (Fig. 3)

To determine the percentage of apoptotic populations, flow cytometric analysis was performed using Annexin-V-FITC and PI staining. The Annexin-V-positive and PI-negative cell population (apoptotic cell death population) was markedly increased in DHLA- and ALA-treated cells. In addition, marked increases in the double-positive cell population (necrotic cell death or terminal stage of apoptotic cell death population) were also detected when cells were treated with DHLA but not with ALA for 24 h (Table 1). This phenomenon was also observed when cells were treated with ALA or DHLA for 12 h (*data not shown*).

Next, whether ALA or DHLA could induce mitochondrial permeability transition (MPT) in HL-60 cells after 12 h of treatment was examined. MitoTracker Orange is a mitochondria-selective reagent and is often used as a mitochondrial dye. The *x*-axis in Fig. 4 represents the fluorescence intensity for the MitoTracker Orange dye. The control and ALA-treated cells retained MitoTracker Orange in their mitochondrial membranes. On the other hand, a pale orange population was observed in DHLA-treated

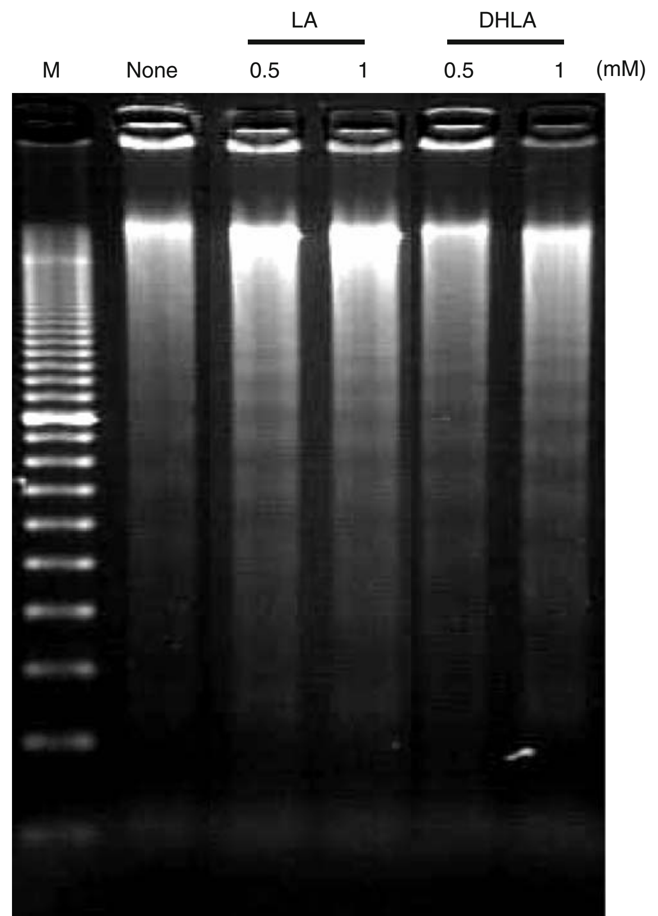

Figure 2. Internucleosomal fragmentation of DNA in HL-60 cells treated with ALA and DHLA. Cells were harvested at 1.0×10^5 cells/ml and cultured for 24 h with or without various concentrations of ALA or DHLA. Fragmented DNA was subjected to 2% agarose gel electrophoresis and detected by staining with SYBR green dye.

cells, which was unable to retain MitoTracker Orange in their membranes. There were no marked differences in histogram pattern among any groups after 6 h of treatment (*data not shown*).

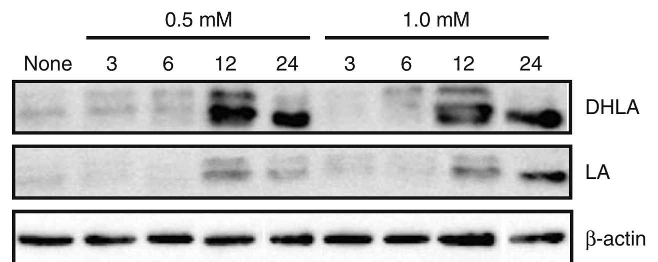

Figure 3. Cleavage of caspase-3 by ALA and DHLA in HL-60 cells. Cells were harvested at 1.0×10^5 cells/ml and cultured for 3, 6, 12, or 24 h with or without 0.5 or 1 mM ALA or DHLA. Cleaved caspase were detected by Western blotting analysis. Cell lysates containing 10 μg of protein were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels and then transferred onto polyvinylidene difluoride Hybond-P membranes. Detection of each protein was performed using an ECL Plus kit.

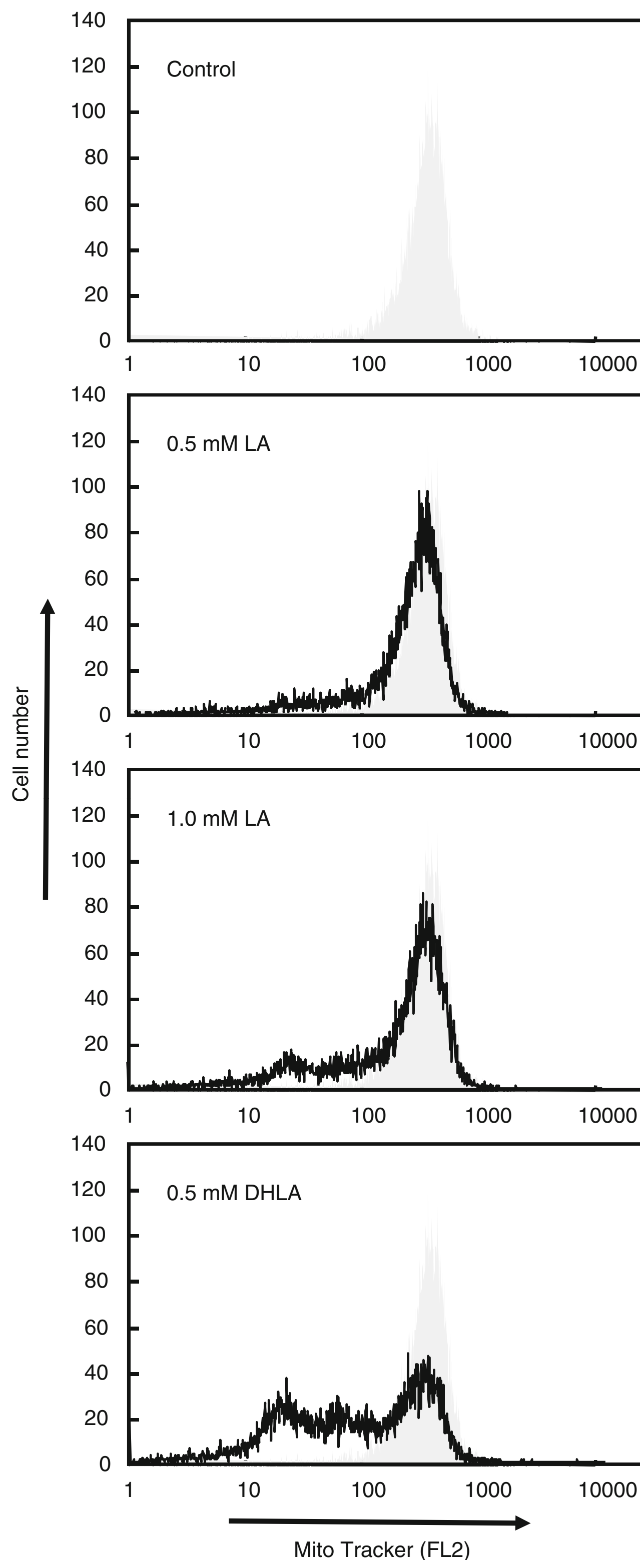

Figure 4. Mitochondrial permeability transition induced by DHLA treatment in HL-60 cells. Cells were harvested at 1.0×10^5 cells/ml and cultured for 12 h with or without 0.5 or 1 mM ALA or 0.5 mM DHLA. At the end of the culture period, cells were stained with MitoTracker Orange. After staining, cells were subjected to flow cytometric analysis. The *filled histogram* shows control.

Next, whether oxidative stress is involved in the cytotoxic activity of ALA and DHLA was examined. As shown in Fig. 5*A*, NAC and Toc were used as antioxidants, but they did not prevent the cytotoxic effects of ALA or DHLA. In addition, ALA did not change the intracellular redox state. On the other hand, 0.5 mM DHLA slightly reduced intracellular ROS level after 12 h of treatment (Fig. 5*B*). There were no marked differences in histogram pattern when cells were treated with ALA or DHLA for 3 h (*data not shown*).

Discussion

Recently, ALA has become the focus of research as an anticancer reagent. Several studies have shown that ALA elicits apoptotic cell death in a number of cancer cells. Intracellular DHLA, a reduced form of ALA, is expected to contribute to various physiological functions of ALA; indeed, it may have higher physiological functions than ALA because of its vicinal thiol groups. Therefore, the cytotoxic potentials of ALA and DHLA were compared in HL-60 cells in the present study. The results of this study showed that cytotoxic activities of 0.5 mM DHLA and 1 mM ALA were comparable and 1 mM DHLA could exert more potent cytotoxic activity than 1 mM ALA. These observations indicated that DHLA exerts a stronger cytotoxic effect than ALA. Therefore, the study next focused on the mechanism of ALA- and DHLA-induced cell death in HL-60 cells.

ALA is known to induce apoptosis in several cancer cell lines via caspase-dependent or caspase-independent pathways (Marsh et al. 2005; Wenzel et al. 2005; Simbula et al. 2007). The results of the present study also showed that ALA induces cell death, internucleosomal DNA fragmentation, and caspase cleavage, which are typical features of apoptotic cell death. In addition, annexin-V/PI staining showed that the apoptotic cell population was significantly increased in ALA-treated cells. These data imply that ALA induces caspase-related apoptotic cell death in HL-60 cells. Moreover, cleavage of caspase-3 and internucleosomal DNA fragmentation were clearly detected in DHLA-treated cells. These data again imply that DHLA activates the caspase-3-related apoptosis pathway. Higher percentages of necrotic or terminally apoptotic cells were detected using annexin-V/PI staining in DHLA-treated cells than in those treated with ALA. These data raise two possibilities: DHLA may induce apoptotic cell death more strongly and rapidly than LA, or DHLA may induce apoptotic and necrotic cell death simultaneously. Recent studies showed that necrosis and apoptotic cell death are not necessarily independent of each other (De Giorgi et al. 2002; Precht et al. 2005).

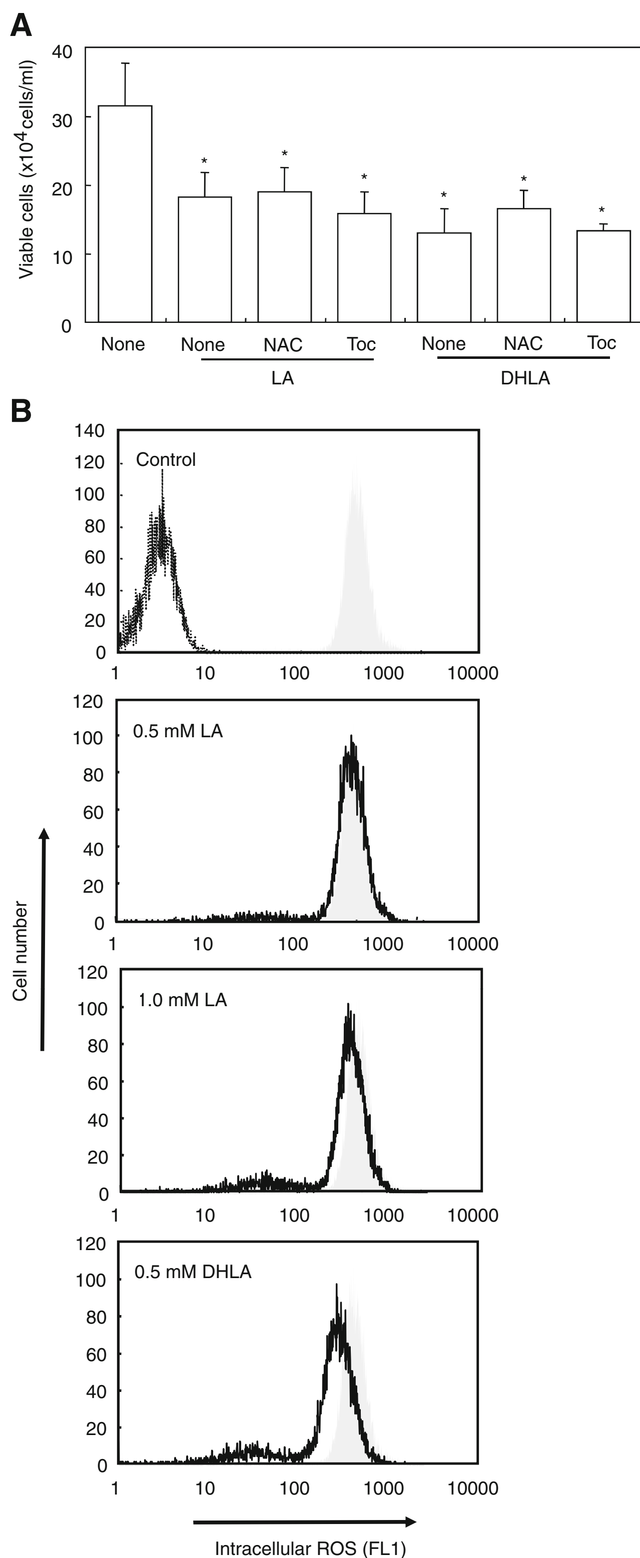

Figure 5. Involvement of intracellular redox state in the cytotoxicity of ALA or DHLA in HL-60 cells. Data are means±SE for three samples. Significant differences from control values are indicated by *asterisks*: **P*<0.05. Cells were harvested at 1.0×10^5 cells/ml and cultured for 12 h with or without antioxidants with ALA or DHLA. Viable cells were counted using the trypan blue exclusion method (*A*). At the end of the culture period, cells were stained with DCFH-DA and then subjected to flow cytometric analysis (*B*). The *dotted line* shows the histogram of unstained control and the *filled histogram* shows control.

MPT is a functional disruption of the mitochondrial inner membrane accompanied with mitochondrial swelling as a key morphological change, which triggers induction of necrotic cell death (Tsujimoto and Shimizu 2007). In addition, MPT triggers various types of cell death in addition to necrosis, such as apoptosis and autophagic cell death (Lemasters et al. 2002; Kim et al. 2003). Interestingly, DHLA was shown to promote MPT more effectively than ALA (Saris et al. 1998). In the present study, experiments using MitoTracker further confirmed that mitochondrial membrane potential was also reduced in DHLA-treated cells. These data indicate that DHLA but not ALA could induce apparent MPT. Although it remains to be determined why DHLA but not ALA could induce MPT, our findings imply a difference in mechanism of action between ALA and DHLA.

The intracellular redox state is involved in the regulation of many intracellular signaling pathways. Especially, oxidative stress such as exposure to reactive oxygen species (ROS) often causes cell death in various cell types (Laurent et al. 2005). Moreover, ROS stress caused by hydrogen peroxide induces apoptotic cell death, but higher level treatment changes the mode of cell death to necrosis (Saito et al. 2006). ALA acts as a pro-oxidant and an antioxidant (Schweizer and Richter 1996; Moini et al. 2002). Indeed, ROS production has been shown to precede ALA-induced apoptotic cell death in several cancer cells (Marsh et al. 2005; Wenzel et al. 2005; Moungjaroen et al. 2006; Simbula et al. 2007). In vitro studies revealed that DHLA-derived ROS production stimulated MPT in rat liver mitochondria (Schweizer and Richter 1996; Morkunaite-Haimi et al. 2003). Therefore, the present study examined whether ALA or DHLA exerted cytotoxicity via stimulation of ROS production. Unexpectedly, no elevation of intracellular ROS level was detected preceding ALA- or DHLA-induced cell death. Moreover, Toc and NAC could not prevent ALA or DHLA cytotoxicity, suggesting that the cytotoxicities of ALA and DHLA progressed in a ROS-independent manner in HL-60 cells.

Recently, it is shown that plasma ALA level reached 10.6–33.8 μg/mL (approximately 50 to 150 μM) at 10–20 min after oral administration of 600 mg of ALA sodium salt in human subjects. (Carlson et al. 2007). Therefore, the concentration used in the present study was higher than the plasma ALA level reported in previous clinical studies.

In conclusion, the results of the present study revealed that DHLA has more potent cytotoxic activity than ALA, and therefore, DHLA is expected to be the more potent cancer chemopreventive agent. ALA is enzymatically converted to DHLA in mitochondria, and therefore, the conversion efficiency is expected to depend on ALA reductase activity. To make the best use of ALA and DHLA as promising cancer chemopreventive agents, it remains to be determined how cellular conversion of ALA into DHLA could contribute to its cancer cell killing activity.

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