Mechanism of α-Lipoic Acid-Induced Apoptosis of Lung Cancer Cells

Involvement of Ca^{2+}

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α-Lipoic acid (LA) shows a protective effect on oxidative stress-induced apoptosis while it induces apoptosis in various cancer cells. Intracellular Ca^{2+} plays a central role in triggering apoptotic pathways. In the present study, we aim to investigate whether LA induces apoptosis in lung cancer cells and whether Ca^{2+} is involved in LA-induced apoptosis. We found that LA decreased cell viability and increased DNA fragmentation of the cells. LA activated the caspase-independent pathway, determined by upregulation of poly(ADP-ribose) polymerase (PARP) and increased the nuclear level of apoptosis-inducing factor and caspase-dependent apoptotic pathway, determined by increased levels of cytochrome *c* and PARP-1 cleavage product. LA-induced apoptotic alterations were inhibited in the cells treated with Ca^{2+} chelator BAPTA-AM. In conclusion, LA induces apoptosis through caspase-independent and caspase-dependent pathways, which is mediated by intracellular Ca^{2+}.

Key words: **α-lipoic acid; apoptosis; lung cancer cells; Ca^{2+}**

Introduction

α-Lipoic acid (LA), also known as thioctic acid, is a naturally occurring compound that is synthesized in small amounts by plants and animals, including humans.[1] Endogenously synthesized LA functions as cofactors for several important mitochondrial enzyme complexes.[2] In addition to the physiological functions of LA, there are increasing scientific and medical interests in potential therapeutic uses of LA. LA acts as an anti- or a proapoptotic agent in various cells. LA prevents oxidant-induced cell death and apoptosis, while LA induces apoptosis in cancer cells.[3–7]

LA has antioxidant activity by quenching a variety of intracellular reactive oxygen species (ROS).[2] On the other hand, LA stimulates mitochondrial Ca^{2+} release and increases the intracellular Ca^{2+} level by oxidizing some protein vicinal thiols.[8,9] Increased Ca^{2+} induces necrosis and/or triggers the controlled pathways of apoptotic cell death.[10]

There are two types of apoptosis.[11] One pathway is a death pathway that traditionally involves Bcl family proteins and caspases (caspase-dependent pathway). Increased Ca^{2+} stimulates mitochondrial stress through activating the Bcl family proteins and initiates apoptosis. Cytochrome *c* is released from the mitochondria of the cells undergoing apoptosis. The other pathway does not involve Bcl/Bax and caspase activation (caspase-independent pathway). In this type of cell death, apoptosis is often triggered by poly(ADP-ribose) polymerase (PARP). PARP is hyperactivated in the caspase-independent pathway.[12] DNA damage by various cell-death signaling, such as Ca^{2+} and ROS, induces hyperactivation and upregulation of PARP and translocation of apoptosis-inducing factor (AIF) into the

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nucleus and initiates apoptosis. Although LA induces apoptosis in several cancer cells, the mechanism of LA-induced apoptosis remains to be investigated.

We aim to investigate whether LA induces apoptosis in lung cancer cells and whether Ca^{2+} is involved in LA-induced apoptosis, by determining cell viability, DNA fragmentation, the levels of AIF, PARP, and cytochrome *c* in the cells, using Ca^{2+} chelator BAPTA-AM. In the present study, A549 cells, a commonly used human lung carcinoma cell line, were employed to elucidate the mechanism of LA-induced apoptosis.

Materials and Methods

A549 cells (human lung carcinoma, ATCC CCL185) were purchased from American Type Culture Collection (Rockville, MD). The cells were grown at 37°C at 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex, Walkersville, MD), and antibiotics (GIBCO). LA and 1,2-bis (2-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) were dissolved in ethanol and dimethylsulfoxide, respectively.

For concentration response of LA for cell viability and DNA fragmentation, the cells were treated with LA (0.5, 1 mmol/L) for 24 h. For time response of LA for the levels of AIF, PARP-1, PARP-1 cleavage product, and cytochrome *c*, the cells were treated with LA (1 mmol/L) for 0, 6, 12, and 24 h. Histone H1 was used for the nuclear control, while actin served as a loading control. To investigate the involvement of Ca^{2+} on LA-induced apoptosis, the cells were treated with or without BAPTA-AM (5, 10 μmol/L) for 2 h. After removing BAPTA-AM from the medium, the cells were treated with LA (1 mmol/L) for 24 h.

Cell viability was determined by the trypan blue exclusion test (0.2% trypan blue). Cells (6×10^4) were seeded onto a 24-well culture plate and then treated with LA for 24 h. The number of viable cells was counted with a hemocytometer, using 0.2% trypan blue.

DNA fragmentation was determined by mononucleosome- and oligonucleosome-bound DNA in the cell lysates, using a sandwich ELISA (cell death detection ELISA plus kit; Roche GmbH, Mannheim, Germany). The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor.

For Western blot analysis, whole-cell extracts were prepared for the detection of PARP-1, PARP-1 cleavage product, cytochrome *c*, and actin. Nuclear extracts were prepared for the detection of AIF and histone H1 by the methods previously described.[13] Briefly, the harvested cells were extracted with lysis buffer (10 mmol/L Tris-HCl, pH7.4, 10% NP-40, and protease inhibitor cocktail) and centrifuged. The supernatants were used for whole cell extracts. To prepare nuclear extracts, the cells were extracted in buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1.5 mmol/L $MgCl_2$, 0.2% NP-40, 1 mmol/L dithiothreitol (DTT), and 0.5 mmol/L phenylmethylsulfonylfluoride (PMSF). The nuclear pellet was resuspended on ice in nuclear extraction buffer (20 mmol/L HEPES, 420 mmol/L NaCl, 0.1 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 25% glycerol, 1 mmol/L DTT, and 0.5 mmol/L PMSF).

The extracts (50 μg protein/lane) were subjected to 8–10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ) by electroblotting. The membranes were blocked using 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.2% Tween 20) for 2 h at room temperature. The proteins were detected with antibodies for PARP-1, actin, AIF (Santa Cruz Biotechnology, Santa Cruz, CA), cytochrome *c* (BD Biosciences Pharmingen, San Diego, CA), and histone H1 (Millipore Corporation, Bedford, MA)

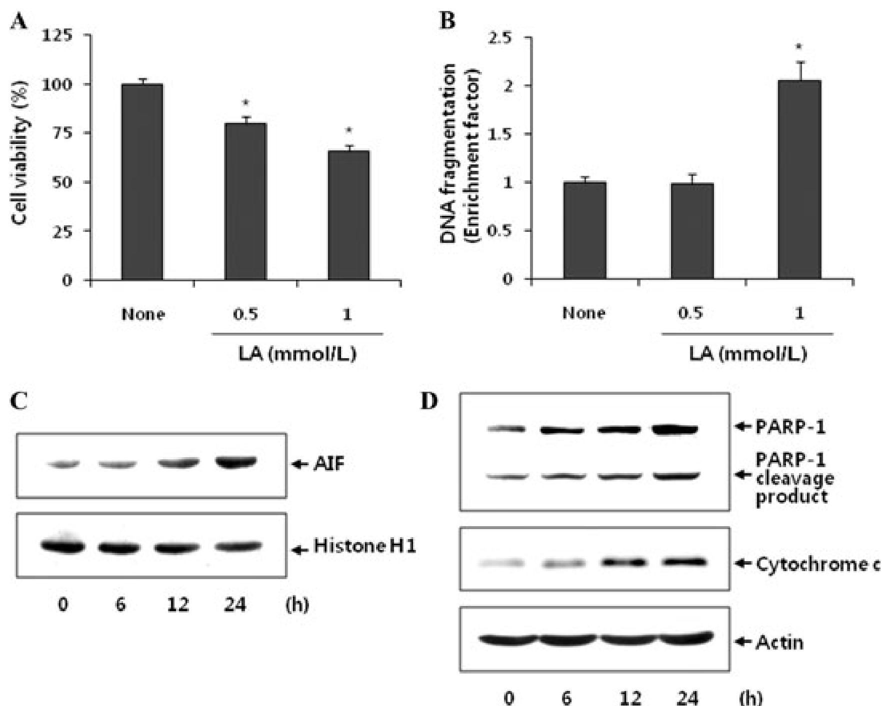

Figure 1. Cell viability, DNA fragmentation, nuclear apoptosis-inducing factor (AIF) level, and levels of poly(ADP-ribose) polymerase (PARP)-1, PARP-1 cleavage product, and cytochrome *c* in α-lipoic acid (LA)-treated A549 cells. (**A**) The cells were treated with LA for 24 h. Viable cell numbers were determined by the trypan blue exclusion assay. Cell viability was expressed as percent cells. (**B**) The cells were treated with LA for 24 h. DNA fragmentation was determined as the content of nucleosome-bound DNA by ELISA. The relative increase in nucleosomes in the cell lysate, determined at 405 nm, was expressed as an enrichment factor. Each bar represents mean ± SE of three separate experiments. *, $P < 0.05$ versus none (cells treated without LA). (**C**) AIF in the nucleus was determined by Western blotting. Histone H1 served as a nuclear marker. The cells were treated with LA (1 mmol/L) for indicated time points. (**D**) The levels of PARP-1, PARP-1 cleavage product, and cytochrome *c* in whole-cell extracts were determined in the cells treated with LA (1 mmol/L) for indicated time points. Actin was used for a loading control.

diluted in TBS-T containing 5% dry milk, and incubated at 4°C for 16 h. After washing with TBS-T, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and visualized by the electrogenerated chemiluminescent (ECL) detection system (Santa Cruz Biotechnology), according to the manufacturer's instructions.

The statistical differences were determined using one-way ANOVA by the Newman–Keuls test. All values were expressed as means ± SE of three separate experiments. $P < 0.05$ was considered statistically significant.

Results

To investigate the effect of LA on apoptosis, viable cell numbers and DNA fragmentation were determined in the cells treated with LA (0.5 or 1 mmol/L) for 24 h (Fig. 1A, B). LA induced a decrease in cell viability dose dependently. LA-induced DNA fragmentation was evident in the cells treated with 1 mmol/L of LA. As shown in Figure 1C, the nuclear level of AIF was increased by LA (1 mmol/L) time dependently, while histone H1, a nuclear control, was not changed by LA. LA increased the level

of PARP-1 and PARP-1 cleavage product in a time-dependent manner (Fig. 1D). These phenomena were in parallel with the increase in the cytochrome *c* level in the cells treated with LA (1 mmol/L).

For the study on possible involvement of Ca^{2+} on LA-induced apoptosis, the cells were pretreated with BAPTA-AM (5, 10 μmol/L) for 2 h and treated with LA (1 mmol/L) for a further 24 h (Fig. 2). DNA fragmentation and protein levels of AIF, PARP-1, PARP-1 cleavage product, and cytochrome *c* were determined in the cells treated with or without BAPTA-AM and cultured with or without LA. LA-induced DNA fragmentation of the cells was significantly inhibited by 10 μmol/L of BAPTA-AM (Fig. 2A). BAPTA-AM inhibited not only PARP-1 induction but also PARP-1 cleavage caused by LA (1 mmol/L) (Fig. 2C). AIF levels in nuclear extracts and cytochrome *c* levels in whole-cell extracts were increased by LA and were inhibited by BAPTA-AM (5, 10 μmol/L) (Fig. 2B, C).

Discussion

In the present study, we showed that LA induced DNA fragmentation and cell death by inducing PARP-1 expression and translocation of AIF into the nucleus. PARP-1 mediates the release of AIF.[12] AIF directly binds DNA and induces large-scale DNA fragmentation.[14,15] Therefore, LA induces apoptosis via the caspase-independent pathway. On the other hand, LA increased levels of both cytochrome *c* and PARP-1 cleavage product, which demonstrates that the caspase-dependent pathway is also involved in LA-induced apoptosis.

In the caspase-independent apoptotic pathway, Bcl/Bax and caspase are not required.[11] Mitochondria release proapoptotic proteins by a distinct mechanism that does not require caspase activation. A key molecule in this type of cell death is PARP-1, which mediates the release of AIF.[12] PARP-1 is an abundant nuclear protein that is involved in the DNA base excision-repair system. In response to DNA damage, PARP-1 activity and expression rapidly increased, resulting in NAD^{+} and ATP depletion and cell death. Therefore, PARP-1 activation might be a key factor that regulates the fate of the cells to either die or survive following DNA damage.[16,17]

Present results showed that LA increased PARP-1 expression, which was inhibited by a Ca^{2+} chelator (BAPTA-AM). The results clearly demonstrate the involvement of Ca^{2+} on LA-induced PARP-1 expression of lung cancer cells. LA was previously shown to stimulate mitochondrial Ca^{2+} release, presumably by oxidizing some protein vicinal thiols.[9] Inositol 1,4,5,-triphosphate (IP_3)-dependent Ca^{2+} mobilization induced PARP-1 activation in brain cortical neurons in the absence of DNA breaks.[18] Other studies showed that Ca^{2+} mediated ROS-induced DNA damage, PARP-1 activation, and cell death.[19,20] In our previous study, LA induced ROS production and apoptosis in lung cancer cells (data not shown). Therefore, further studies should be performed to investigate the relation between Ca^{2+} and ROS in the LA-induced apoptotic mechanism of lung cancer cells.

AIF is a key mediator of cell death downstream of PARP-1 activation because PARP-1 stimulates translocation of AIF into the nucleus.[16,17,21] In response to apoptotic stimuli, AIF is released from the mitochondria and translocates into the nucleus. It becomes an active executioner of the cells.[11] LA-induced PARP-1 expression may accelerate release of AIF from mitochondria and subsequent nuclear translocation. AIF localized in the nucleus initiates chromatin condensation and large-scale (50 kb) DNA fragmentation. Consequently, AIF plays a central role in the regulation of the caspase-independent apoptotic pathway.[11]

The cytochrome *c*-mediated caspase activation pathway is well established.[22] Upon sensing a variety of apoptotic stimuli, cytochrome *c* is released from mitochondria and triggers

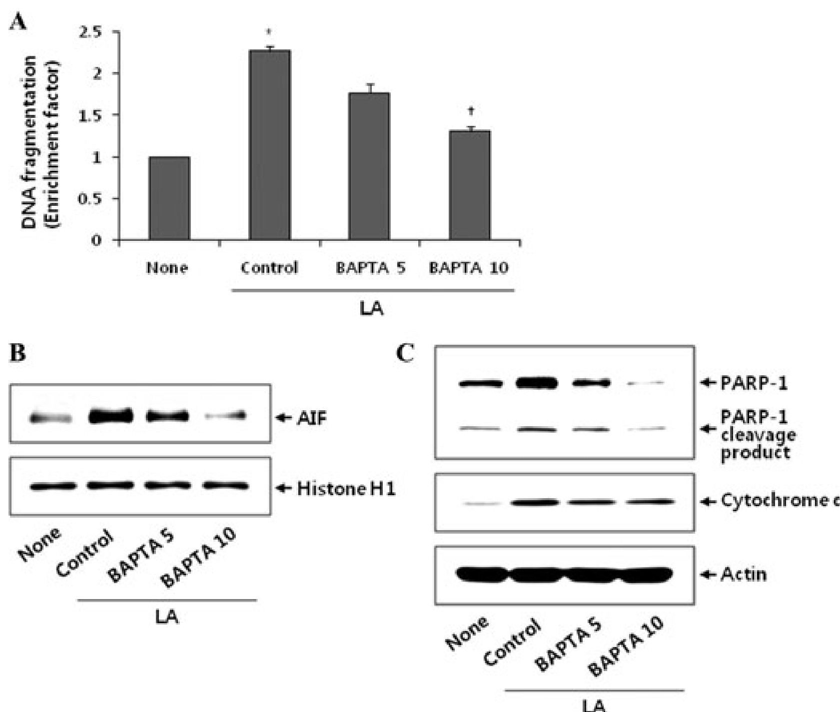

Figure 2. DNA fragmentation, nuclear AIF level, and levels of PARP-1, PARP-1 cleavage product, and cytochrome *c* in A549 cells treated with or without BAPTA-AM and cultured in the presence of LA. (**A**) The cells were pretreated with BAPTA-AM for 2 h. After removing BAPTA-AM from the medium, cells were treated with LA (1 mmol/L) for a further 24 h. Each bar represents mean ± SE of three separate experiments. *, $P < 0.05$ versus none (cells treated without LA); †, $P < 0.05$ versus control (cells treated with LA without BAPTA-AM). (**B**) AIF in the nucleus was determined by Western blotting. Histone H1 served as a nuclear marker. The cells were pretreated with BAPTA-AM (5, 10 μmol/L) for 2 h and then treated with LA (1 mmol/L) for 24 h after removing BAPTA-AM from the medium. (**C**) The levels of PARP-1, PARP-1 cleavage product, and cytochrome *c* in whole-cell extracts were determined in the cells pretreated with BAPTA-AM (5, 10 μmol/L) for 2 h and treated with LA (1 mmol/L) for a further 24 h. Actin was used as a loading control.

formation of the apoptosome. The apoptosome subsequently forms an active holoenzyme with procaspase-9 and activates downstream executioner caspases, such as caspase-3, which eventually lead to programmed cell death.[22] Activated caspase-3 then cleaves whole PARP-1 to prevent energy depletion and subsequent necrotic cell death.[23] Therefore, PARP-1 cleavage product is used as an activated caspase-3 marker. In the present study, LA increased the expression of cytochrome *c* and PARP-1 cleavage products, indicating that the caspase-dependent pathway is activated in LA-induced apoptosis of A549 cells. LA stimulates mitochondrial Ca^{2+} release.[8] Elevating intracellular Ca^{2+} concentration encourages mitochondrial membrane potential transition that releases cytochrome *c* from mitochondria to cytosol and triggers Ca^{2+}-modulated apoptosis.[10] Accumulating evidence supports the present results showing that Ca^{2+} chelator inhibited LA-induced cytochrome *c* and PARP-1 cleavage products in lung cancer cells. In addition, lung cancer A549 cells are non-small-cell lung cancer cells, which are resistant to chemotherapy-induced apoptosis.[24] Therefore, LA may be beneficial for the treatment of lung cancer patients who show chemotherapy resistance.

In the present study, we demonstrate that LA induces apoptosis through the caspase-independent pathway (nuclear translocation of AIF and PARP-1 induction) and caspase-dependent pathway (cytochrome *c* release and PARP-1 cleavage), which is mediated by intracellular Ca^{2+}.

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Conflicts of Interest

The authors declare no conflicts of interest.

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