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Alpha-lipoic acid induces apoptosis in hepatoma cells via the PTEN/Akt pathway

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Abstract We report here that alpha-lipoic acid (α-LA), a naturally-occurring antioxidant, scavenges reactive oxygen species (ROS) followed by an increase in apoptosis of human hepatoma cells. Apoptosis induced by α-LA was dependent upon the activation of the caspase cascade and the mitochondrial death pathway. α-LA induced increases in caspase-9 and caspase-3 but had no significant effect on caspase-8 activity. Apoptosis induced by α-LA was found to be mediated through the tensin homologue deleted on chromosome 10 (PTEN)/Akt pathway. Prior to cell apoptosis, PTEN was activated and its downstream target Akt was inhibited. Our findings indicate that increasing ROS scavenging could be a therapeutic strategy to treat cancer.
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Keywords: α-Lipoic acid (α-LA); Reactive oxygen species (ROS); Apoptosis; Caspase; PTEN, Akt (PKB)

1. Introduction

Alpha-lipoic acid (α-LA) is a naturally-occurring co-factor found in a number of multi-enzyme complexes regulating metabolism [1]. α-LA or its reduced form dihydrolipoic acid (DHLA) is a powerful antioxidant, it can scavenge various ROS including superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen [1,2]. α-lipoic acid has been reported to induce apoptosis in various tumor cell lines such as colon cancer [3], human squamous cell carcinoma cell line [4] and human lung epithelial cancer cells [5]. However, the molecular mechanisms underlying the apoptotic effect of α-LA are not well understood.

Reactive oxygen species (ROS) are emerging as critical signaling molecules [6]. Under normal conditions, ROS are maintained within narrow boundaries by scavenging systems. Unlike normal cells, tumor cells survive in a specific redox environment where ROS levels are elevated. Increased ROS levels contribute to enhanced cell proliferation and suppression of apoptosis in cancer cells [7]. Previous studies have shown that low dose exogenous ROS can promote tumor cells proliferation [8]. Increasing ROS scavenging, thereby dampening ROS signaling could inhibit tumor growth and induce tumor cell apoptosis.

Phosphatidylinositol 3-kinase (PI3K) and its effector protein kinase B (PKB/Akt) have been implicated as critical mediators of survival [9,10]. We have previously described that ROS are involved in cell proliferation through regulating Akt (PKB) [11,12]. Therefore, the antioxidant α-LA could also target the Akt pathway.

The purpose of this study was to investigate the molecular mechanisms regulating α-LA-mediated apoptosis using SMMC-7721 hepatoma cells. We examined the effect of α-LA on hepatoma cell apoptosis and the pathways involved.

2. Materials and methods

2.1. Materials

2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Calbiochem. Caspase-3 fluorogenic substrate (Ac-DEVD-AMC), caspase-8 fluorogenic substrate (IETD-AMC) and caspase-9 fluorogenic substrate (LEHD-AMC) were from Alexis Biochemicals. Anti-Akt, anti-phospho-Akt (Ser-473), anti-tensin homologue deleted on chromosome 10 (PTEN), and anti-phospho-PTEN antibodies (Ser-380/Thr-382/-383) were purchased from Cell Signaling. Anti-β-actin and anti-horseradish peroxidise (HRP)-conjugated antibodies were from Santa Cruz Biotechnology. BCA protein assay reagent was from PIERCE. Culture media and fetal bovine serum were from Gibco/BRL.

2.2. Cell culture

The 7721 human hepatoma cell line was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium supplement with 10% fetal calf serum, 200 μg/ml penicillin, 300 μg/ml glutamine. α-LA dissolved in 1 N NaOH and neutralized in medium, was added to the culture media to the final concentrations specified in the text. Control cells were treated with an equivalent amount of the solvent alone.

2.3. Measurement of intracellular ROS

The intracellular ROS production was measured as described before [11]. Briefly, the cells were incubated 30 min at 37 °C with 10 μM 2′,7′-DCFH-DA in PBS. After washing out the excess probe, the cells were

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Abbreviations: α-LA, α-lipoic acid; DCFH-DA, dichlorofluorescein diacetate; DHLA, dihydrolipoic acid; PI3K, phosphoinositide 3-kinase; PtdIns(3,4,5)P_3, phosphatidyl inositol-3,4,5-triphosphate; PtdIns(4,5)P_3, phosphatidyl inositol-4,5-triphosphate; PKB/Akt, protein kinase B/Akt; PTEN, tensin homologue deleted on chromosome 10; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling

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transferred to a fluorometer cuvette, and the fluorescence was recorded at 490 nm excitation and 530 nm emission in a Hitachi F-3000 fluorescence spectrophotometer. Intracellular ROS production was calculated from an H_2O_2 standard curve.

2.4. Assessment of cell viability

Cells were trypsinized with 0.25% trypsin and harvested at different time point. Cell number was counted using a haemocytometer by adding 0.2% trypan blue which stains the cytoplasm of dead cells but not live cells. Live cells were counted using this trypan blue exclusion method.

2.5. Apoptosis assay and flow cytometry

SMMC-7721 cell apoptosis was assessed by terminal deoxynucleotidyl transferase (Tdt) mediated dUTP Nick End Labelling (TUNEL) analysis using flow cytometry. 10^6 cells were washed twice in PBS and fixed in 1% paraformaldehyde in PBS at 4 °C for 30 min followed by post-fixation in 70% ethanol for 30 min. Cells were subsequently stained using the ApoBrdu kit (Phoenix Flow systems) according to the manufactures protocol. 450 μl of RNase/propidium iodide solution was subsequently added and incubated for 30 min at room temperature. Samples were analyzed on a Becton Dickinson FACS Calibur (San Jose, USA).

2.6. Caspase activity assay

Caspase activity was determined by fluorometric assay using the enzyme substrate Ac-DEVD-amino-4-methyl coumarin (AMC) for caspase-3, IETD-AMC for caspase-8 and LEHD-AMC for caspase-9, which are specifically cleaved by the respective enzymes at the Asp residue to release the fluorescent group, AMC. Cells were processed according to the manufactures protocol. 5×10^5 cells were centrifuged. Cell pellets were re-suspended in PBS and subsequently 12.3 μl of substrate was added. Following incubation at 37 °C for 60 min, the liberated fluorescent product was determined fluorometrically at the excitation and emission wavelengths of 488 and 576 nm, respectively.

2.7. Western blot analysis of Akt and PTEN

Cell extracts were prepared by incubating the cells in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% triton X-100, 1 mM PMSF, 2.5 μg/ml leupeptin, 1% aprotinin, 10 mM sodium Vanadate, 10 mM β glycerol phosphate) for 30 min on ice. Equal amount of proteins per sample (30 μg) were resolved on a 10% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The transferred membranes were blocked for 1 h in 5% non-fat dry milk in 0.1% Tween 20 in Tris-buffered saline (TBS) and incubated with appropriate primary antibodies at 4 °C overnight. Membranes were washed three times with 0.1% Tween 20 in TBS for 10 min and incubated with secondary antibodies for 1 h at room temperature. Antibody binding protein bands were detected by using enhanced chemoluminescence reagent (ECL) and X-ray film.

3. Results

3.1. α-LA scavenges ROS in SMMC 7721 human hepatoma cells

We examined the effect of α-LA on ROS generation in hepatoma cells. Cellular ROS was examined using the fluorescent probe DCF-DA, which detects hydroperoxide and $O_2^{\cdot -}$. The validation of the ROS detection by DCF fluorescence was verified by ESR method as described in our previous work [12]. The changes in DCF fluorescence intensity of probes reflected the variation in intracellular ROS in our experiment (data were not shown). Fig. 1 shows that treatment of SMMC-7721 cells with 5 mM α-LA caused a rapid suppression of ROS level by 6 h which remained decreased for 18 h, as indicated by the decrease in DCF fluorescence intensity. This suggests that α-LA was able to scavenge intracellular ROS.

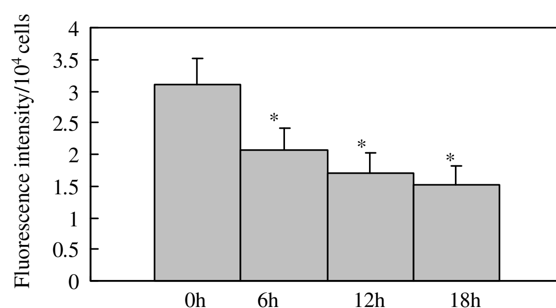

Fig. 1. α-LA scavenges ROS in SMMC-7721 human hepatoma cells. Plots show fluorescence intensity over 10^4 cells at the indicated time after treatment. Cells were treated with 5 mM α-LA for 6, 12, and 18 h. After treatment the cells were loaded with DCFH-DA and fluorescence was recorded at 490 nm excitation and 530 nm emission as described in Section 2. Values are means ± S.D. ∗, $P < 0.05$ versus control.

3.2. α-LA inhibits cell growth in SMMC-7721 human hepatoma cells

SMMC-7721 hepatoma cells were cultured in the presence or absence of various concentration of α-LA for 4 days. Cell growth was assessed at 24 h intervals by live cell counts using the trypan blue exclusion method. Treatment with increasing concentrations of α-LA (1, 2.5 and 5 mM) resulted in a dose- and time-dependent decrease of viable cell number compared to control (Fig. 2). In particular, a significant inhibition of cell growth 2 days after treatment with the 2.5 and 5 mM concentrations of α-LA was observed.

3.3. α-LA induces apoptosis in SMMC-7721 human hepatoma cells

In order to understand whether the inhibition of cell growth is the consequence of apoptosis, we analyzed the apoptosis in the cells treated with 5 mM α-LA at different times by TUNEL as described in Section 2. As shown in Fig. 3, treatment of SMMC-7721 cells with 5 mM α-LA caused a time-dependent increase of apoptotic cells from day 3 onwards. By day 3 18% of the cells were apoptotic compared to 1.3% in the control study. By day 4 the percentage of apoptotic cells had reached 65%.

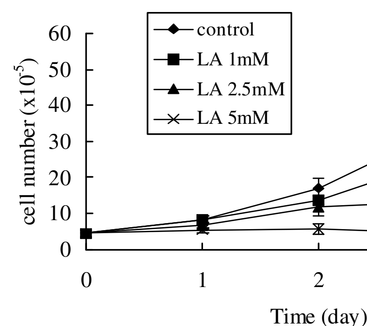

Fig. 2. Effect of α-LA on the proliferation of SMMC-7721 human hepatoma cells. SMMC-7721 hepatoma cells were treated with 1, 2.5, and 5 mM α-LA. Cells were harvested at 1–4 days after treatment. Viable cells were assessed by live cell counting using trypan blue exclusion method. The values represent the means ± S.D. from three separate experiments.

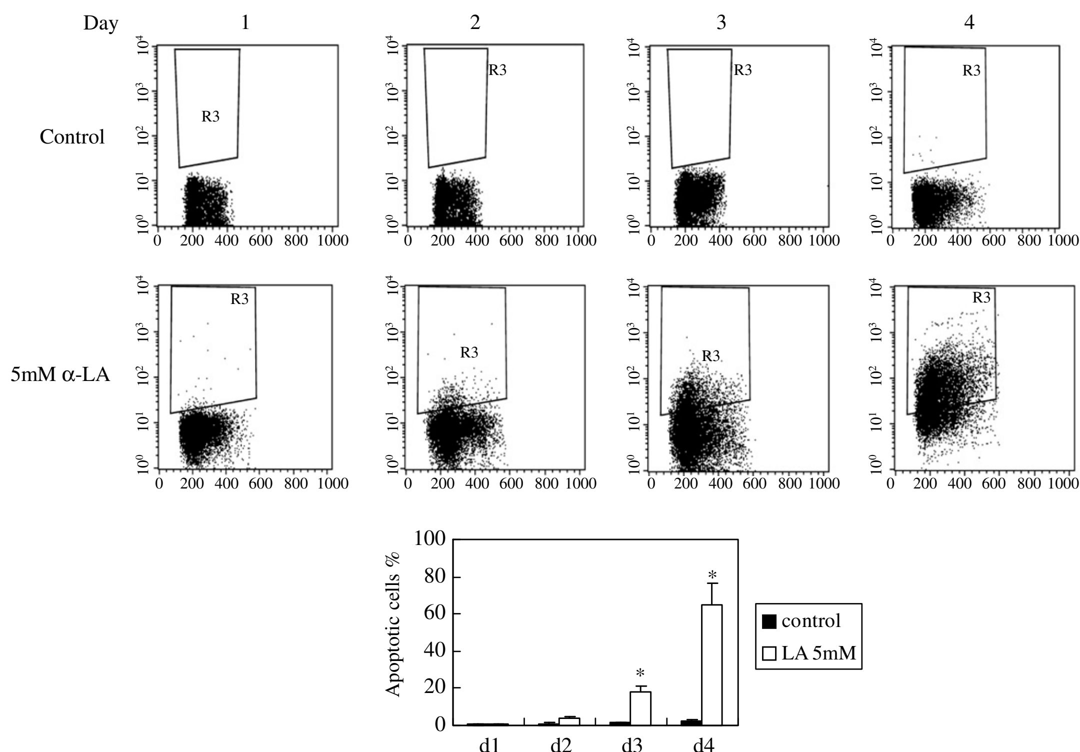

Fig. 3. α-LA induces apoptosis in SMMC-7721 human hepatoma cells. SMMC-7721 hepatoma cells were cultured for 1– 4 days with 5 mmol/L α-LA. Cells were harvested at 1–4 days after treatment. Apoptosis was evaluated by TUNEL as described in Section 2. Results are the means ± S.D. from three separate experiments. $^{*}P < 0.05$ compared to control.

3.4. Effect of α-LA on caspase activation in SMMC 7721 hepatoma cells

Apoptosis is a process modulated by the caspase family of enzymes [13,14]. We therefore examined whether apoptosis induced by α-LA was dependent upon the activation of the caspase cascade. The activities of caspases (3, 8 and 9) were detected following 3 days of treatment by high dose of 5 mM α-LA. Caspase activity assays showed that α-LA induced a 4.2-fold increase in caspase-3 and 6.3-fold increase in caspase-9, but had no significant effect on caspase-8 activity (Fig. 4). It is known that caspase-9 mediates the intrinsic (mitochondrial) pathway of apoptosis and caspase-8 mediates the extrinsic (death receptor) pathway [15,16]. Therefore the results suggest that the major pathway of apoptosis induction by α-LA is the mitochondrial death pathway.

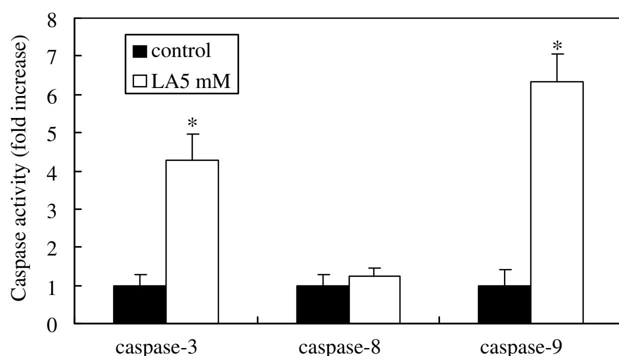

Fig. 4. Effect of α-LA on caspase activation in SMMC-7721 human hepatoma cells. SMMC-7721 hepatoma cells were harvested following 3 days of treatment by 5 mM α-LA. The activities of caspases (3, 8, and 9) were measured using fluorogenic substrate assay as described in Section 2. Results are the means ± S.D. from three separate experiments. $^{*}P < 0.05$ compared to control.

3.5. α-LA causes Akt inhibition associated with PTEN activation

Akt (PKB), a serine–threonine protein kinase, is very important in regulating cell survival and prevention of apoptosis in tumors [17]. We examined whether α-LA-induced caspase activation could be associated with downregulation of Akt. As shown in Fig. 5A, treatment of SMMC 7721 hepatoma cells with α-LA caused an initial downregulation in the levels of the phosphorylated form of Akt at 12 h and a significant decrease after 48 h of treatment. The total Akt had no significant change up to 72 h. α-LA-induced apoptosis appears to be accompanied inhibition of Akt activity. It is known that Akt phosphorylation of caspase-9 leads to inhibition of its protease activity [18]. The data suggests that downregulation of Akt activity by α-LA may account for α-LA-induced caspase activation and apoptosis.

Akt is the main downstream target of PI3K. We then tested whether the decrease of Akt activity might be caused by a decreased PI3K activity. However, PI3K activity had no significant change in response to α-LA (data were not shown). Thus, Akt inactivation was unlikely because of a decrease in PI3K. Then we examined if the phosphatase PTEN, which is a negative regulator of Akt, might cause the downregulation of Akt activity. As shown in Fig. 5B, α-LA caused a time-dependent activation of PTEN activity. PTEN phosphorylation at Ser-380 and Thr-382/-383 was initially elevated at 12 h and continue to increase until 72 h. The PTEN phosphorylation state

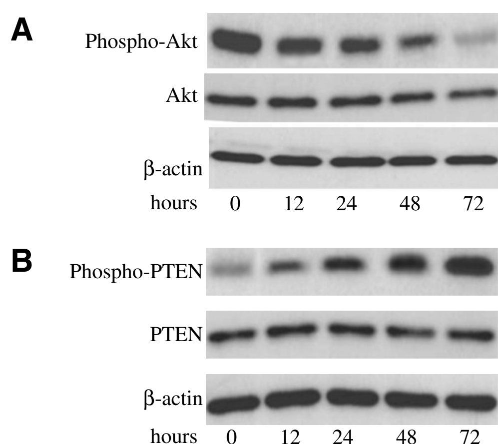

Fig. 5. α-LA causes inactivation of Akt and activation of PTEN. Cells were treated with 5 mM α-LA for the indicated period. (A) Effect of α-LA on Akt expression and phosphorylation. Protein extracts were blotted using anti-phospho-Akt (Ser 473), anti-Akt and anti-β-actin antibodies as described in Section 2. (B) Effect of α-LA on PTEN expression and phosphorylation. Protein extracts were blotted using anti-phospho-PTEN (Ser-380/Thr-382/-383), anti-PTEN, and anti-β-actin antibodies as described in Section 2. The present blots are representative of three experiments.

was inversely correlated with the degree of Akt phosphorylation. This suggests that α-LA, through activating PTEN, negatively regulated Akt.

4. Discussion

Growing evidence shows that ROS plays a critical role in various cancers[19,20]. Cancer cells usually have elevated levels of ROS and downregulation of ROS scavengers and antioxidant enzymes. Antioxidant treatment could increase antioxidative activity and induce cancer cell apoptosis [21]. α-Lipoic acid is one of the important naturally occurring antioxidants that are synthesized by plants and animals [1]. It has been reported that α-LA has a beneficial effect in patients with advanced cancer through increasing glutathione peroxidase activity and reducing oxidative stress [22]. Recent studies have also shown that α-LA induces apoptosis in some cancer and transformed cell lines [3,4,23]. However, the mechanisms by which α-LA induces cancer cell apoptosis is unclear.

To test whether α-LA can also induce apoptosis in human hepatoma cells and to determine the mechanisms leading to this, we first characterized the cellular ROS level and apoptosis response to α-LA treatment in SMMC-7721 cells. Our results demonstrate that α-LA is able to scavenge ROS in SMMC-7721 human hepatoma cells. The reduction of ROS by α-LA was followed by hepatoma cells growth arrest and apoptosis. Five millimoles α-LA caused a significant reduction of ROS for at least 18 h, followed by significant growth inhibition from day 2, and apoptosis from day 3.

Apoptosis can be initiated through an extrinsic pathway, also referred to as the death receptor pathway; and an intrinsic pathway, also referred to as the mitochondrial pathway. We further investigated by which apoptotic pathway α-LA induced apoptosis in hepatoma cells. The principal executioners of apoptotic cell death are caspase proteases which belong to a family of cysteine proteases. The extrinsic pathway is associated with activation of caspase-8, and the mitochondrial pathway with activation of caspase-9. The two pathways converge into caspase-3 activation and trigger apoptosis [15,16,24]. Our results show that apoptosis induced by α-LA was dependent upon the activation of the caspase cascade. α-LA induced increase in caspase-3 and caspase-9 but had no significant effect on caspase-8 activity. The ability of α-LA to activate caspase-9 and inability of α-LA to activate caspase-8 suggests that their apoptotic effect is mediated through the mitochondrial death pathway.

The Akt (PKB) pathway is a well-known signal transduction pathway mediating growth promotion and cell survival signaling [9,25]. Our results show that α-LA inhibited Akt activity. Since Akt phosphorylates and inhibits the protease activity of caspase-9 [18], inactivation of Akt by α-LA could result in activating caspase-9 and trigger apoptosis. It is known that Akt activity can be positively regulated by PI3K or negatively regulated by PTEN. Sometimes Akt can also be directly activated by selectively phosphorylation at Ser-473, a residue critical for maximal activation [26]. In order to understand the mechanism whereby α-LA induced Akt inactivation, both PI3K and PTEN activities were investigated. The results suggest that PI3K was unlikely to have contributed to Akt activation. PTEN activity was inversely correlated with the degree of Akt activity, which is consistent with the negative regulatory role of PTEN in Akt signaling [27]. This indicates that apoptosis induced by α-LA was mediated through the PTEN/Akt pathway.

PTEN is a phosphatidyl inositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) phosphatase which dephosphorylates of the three position PtdIns(3,4,5)P_3 to produce phosphatidyl inositol-4,5-triphosphate (PtdIns(4,5)P_2), thus direct antagonizing PI3K signaling. ROS is known to be able to inactivate protein phosphatases by its oxidative activity. For example, PTEN phosphatase activity was reported to be oxidatively inhibited by ROS [28]. In this regard, scavenging cellular ROS could activating PTEN, thus inactivating PI3K signaling and promoting apoptosis. As the results we present show, the decrease of Akt levels, the increase of PTEN and the induction of the apoptotic process induced by α-LA may be due to the antioxidative effect of α-LA.

At present, the role of ROS in cancer treatment has been a controversial issue. Both stimulation and suppression of ROS have been reported to induce cancer cell apoptosis. Some reports suggest that α-LA does not have antioxidant effects or even may have pro-oxidant properties that induce apoptosis by increasing ROS levels [3,5,29]. Our data show that α-LA acted as an antioxidant. Depending on the dose, the time of exposure, the type of cells and the experimental conditions, α-LA may act differently [30]. Our results suggest that α-LA scavenged intracellular ROS, thus affecting the cellular redox environment in hepatoma cells. It is known that cancer cells have an elevated level of ROS and that cellular ROS are required for cancer cell growth. Scavenging ROS by α-LA thereby dampened ROS signaling and depressed tumor growth. In this respect, modulation of ROS by reducing ROS levels could confer a therapeutic benefit in cancer treatment. Given that cancer cells have inherently higher levels of ROS than normal cells, this property can be exploited to selectively kill cancer cells but not harm normal cells. Indeed recent studies have shown that α-LA induces apoptosis in some cancer and transformed cell lines but not in non-transformed cell lines [3,4]. Our results also show that 5 mM α-LA was not harmful to normal L02 li-

ver cells (data are not shown). This suggests that α-LA is a potential candidate for cancer treatment.

Taken together, our findings suggest inhibition of ROS generation by α-LA effectively induced apoptosis in hepatoma cells. α-LA, through scavenging ROS, inhibits PI3K signaling and induces mitochondrial pathway mediated apoptosis.

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