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Alpha-eleostearic acid induces autophagy-dependent cell death through targeting AKT/mTOR and ERK1/2 signal together with the generation of reactive oxygen species

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ABSTRACT

Alpha-eleostearic acid (α-ESA, 9Z11E13E-18:3), a linolenic acid isomer with a conjugated triene system, is a natural and biologically-active compound that has been shown to possess potent anti-tumor properties. Herein, we demonstrate α-ESA induced apoptosis and autophagy with reactive oxygen species (ROS) generation in HeLa cells. Treatment with α-ESA caused inhibition of phosphorylated (p)AKT and elongated the sub G1 phase in the cell cycle, indicating induction of apoptosis. Autophagy was also induced by α-ESA treatment, causing low pAKT and pP70S6K activities, increasing pERK1/2 and leading to a higher conversion rate of LC3 I to LC3 II compared to that of the control. The autophagy was further confirmed by fluorescence microscopy and flow cytometry through monodansylcadavarine (MDC) staining. It appears that the role of autophagy is a protective mechanism against cell death in α-ESA-treated HeLa cells. Subsequently, we found that treating HeLa cells with α-ESA induced the generation of reactive oxygen species (ROS). The phosphorylation of P70S6K, downstream of mTOR signaling, and AKT were further reduced by pretreatment with *N*-acetyl-L-cysteine (NAC), an ROS scavenger, whereas the phosphorylation of ERK1/2 and the conversion of LC3 I to LC3 II were further enhanced. As a result, the blocking of the action of ROS promoted α-ESA-induced apoptosis and autophagy. Taken together, our results indicate that the generation of ROS by α-ESA treatment impedes the progress of apoptosis and excessive autophagy formation which takes part in cell death, thus impeding death promotion.

Introduction

Conjugated fatty acids, modulators of the cellular production of reactive oxygen species (ROS) [1,2], have been suggested as therapeutic agents to improve human health. The positive benefits associated with conjugated fatty acids appear to have a close association with the molecular characteristics of conjugated double bonds [3]. In the past few years it has been reported that cis-9, trans-11, or trans-13 conjugated linolenic acids, for example alpha-eleostearic acids (α-ESA) that are mainly obtained from the seed oils of the tung plant (*Aleurites fordii*), have been adopted for alternative cancer therapies [4]. Apoptosis, type I programmed cell death, plays a crucial role in the development and homeostasis of multicellular organisms and is characterized by the condensation of cytoplasm and nucleus, DNA fragmentation, chromatin merging in the nuclear periphery, cell contraction, membrane blebbing, and cell phagocytosis [5,6]. Apoptosis is negatively regulated by the AKT signaling pathway, which phosphorylates BAD, resulting in the inhibition of pro-apoptotic Bax protein through the activation of anti-apoptotic Bcl-2/Bcl-xl proteins [7]. Autophagy, a physiologic process involved in routine turnover of cell constituents, is regulated by AKT/mammalian targets of rapamycin (mTOR) and MAPK, such as the ERK1/2 signaling pathway [8]. Autophagy is a temporary survival mechanism formed during starvation where self-digestion becomes an alternative energy source; at the same time it is also important in the induction of tumor cell death [9]. For instance, excessive autophagy will also inevitably trigger autophagic cell death or 'type II programmed cell death' [10]. However, it is still under debate whether chemotherapy-induced autophagy in tumor cells is a protective response or a

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process to promote cell death [8]. ROS, including superoxide anions (O_2.–), hydrogen peroxide (H_2O_2), and hydroxyl radicals (.OH), act as signaling intermediates [11] and can initiate both apoptosis and autophagy [12,13], however, their precise roles during apoptosis and autophagy remain controversial. In this study we report that α-ESA induced apoptosis and autophagy and caused ROS generation, impeding key signals of apoptosis and autophagy, which include AKT/mTOR and ERK1/2.

Materials and methods

Cell culture and reagents. HeLa, or human cervix carcinoma cells, were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Invitrogen, Grand Island, NY) at 37 °C and 5% CO_2. For the experiments, HeLa cells were incubated in serum-deprived DMEM supplemented with 0.1% FBS for 12 h before being treated with α-ESA. The α-ESA (purity over 95%) was provided by Lipozene Inc. (Pyeongteak, South Korea) and was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemical, St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-metyhyladenine (3-MA), *N*-acetyl-L-cysteine (NAC), propodium iodide (PI) and monodansylcadavarine (MDC) were purchased from Sigma-Aldrich Chemical. Antibodies against phospho-AKT (Ser473), AKT, phospho-P70S6K (Thr389), P70S6K, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 and LC3 were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated (HRP) secondary antibodies and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell viability assay. HeLa cells were plated in 96-well flat bottom plates at a density of 2×10^4 cells/ml. After 24 h of incubation, the cells were incubated in DMEM supplemented with 0.1% FBS for 12 h and treated with growth media containing α-ESA for an additional time period as indicated, following which MTT solution (0.5 mg/ml) was added for 3 h at 37 °C in a humidified incubator with 5% CO_2. The medium were removed, and then formazan dye was solubilized with DMSO and quantified by a spectrophotometer (Molecular Devices Inc. Sunnyvale, CA) at 550 nm.

Cell cycle analysis. To examine apoptosis, 2×10^5 cells/ml HeLa cells were seeded onto six-well culture dishes and incubated for 24 h in a humidified incubator at 5% CO_2 and 37 °C. The cells were then incubated in serum-deprived DMEM supplemented with 0.1% FBS for 12 h and treated with 75 μM α-ESA in growth media for the indicated time periods. The cells were harvested and washed three times with PBS and fixed with ice-cold 70% ethanol at 4 °C for 2 h. The cells were then stained with PI solution (0.5 ml PBS, RNase 10 μg/mL, 0.05% Triton-X 100, and 50 μg/L of PI). DNA content was analyzed by flow cytometry using FACSCalibur and Cell Quest software (BD Biosciences, CA).

Western blot analysis. HeLa cells were grown to 90% confluence on 6-cm culture dishes in DMEM supplemented with 10% FBS and 1% antibiotics, then incubated for 12 h in DMEM supplemented with 0.1% FBS. The cells were treated with various concentrations of α-ESA with or without NAC in growth media for the indicated time periods. The cells were scraped, and pellets were disrupted with RIPA lysis buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris-HCl (pH 7.4), protease inhibitor cocktail (Roche, Mannheim, Germany) and lysed on ice for 15 min. After centrifugation at 22,000*g* for 4 min at 4 °C, the amount of protein in the supernatant was determined by protein assay (Bio-Rad, Hercules, CA). Samples were stored at −70 °C until analysis. Samples were mixed with an equal amount of Laemmli sample buffer (Bio-Rad) and boiled at 100 °C for 5 min, separated by 10–15% SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF) transfer membrane (Amersham Biosciences, Piscataway, NY). Membranes were blocked with 5% non-fat milk in TBST (TBS with 0.05% Tween 20) at room temperature for 2 h. The membrane was washed and incubated with the appropriate primary antibody at 4 °C overnight. The membrane was then washed and incubated with a HRP-conjugated secondary antibody for 1 h at room temperature. The immunoblots were visualized by enhanced Chemiluminescence PLUS ™ (GE Healthcare, Buckingham, UK).

MDC staining. To observe autophagy formation, HeLa cells were grown on glass cover-slips for 24 h in a humidified incubator at 5% CO_2 and 37 °C. The cells were then incubated in serum-deprived DMEM supplemented with 0.1% FBS for 12 h. After incubation with 75 μM α-ESA for 12 h, the cells were treated with 0.05 mM monodansylcadavarine (Sigma-Aldrich Chemical) at 37 °C in 5% CO_2 for 60 min. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min. The cellular changes in fluorescence were observed under fluorescence microscope (Olympus, Tokyo). The fluorescence intensity was also analyzed by flow cytometry (BD Biosciences).

Transmission electron microscopic test. To detect autophagy formation in α-ESA-treated HeLa cells, we performed an ultrastructural analysis according to the procedure described previously [14]. Briefly, after being treated with 75 μM α-ESA for 12 h, HeLa cells were harvested and washed twice with PBS, then fixed with ice-cold glutaraldehyde (3% in 0.1 M cacodylate buffer, pH 7.4) for 30 min. The samples were post-fixed with 1% OsO_4 in the same buffer for 1 h and then subjected to electron microscopic analysis. Representative areas were chosen for ultra thin sectioning and were observed with a JEM 1010 transmission electron microscope (JEOL, Peabody, MA).

ROS measurement. ROS levels were determined using flow cytometry analysis. Briefly, cells were treated with 75 μM α-ESA in the presence or absence of 5 mM NAC and incubated with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCF-DA; Invitrogen) for 30 min. Then the cells were harvested and washed three times with PBS. The cells were suspended in PBS and were analyzed by flow cytometry (BD Biosciences).

Statistical analysis. Data was expressed as means ± standard deviation (S.D.). Statistical comparisons were made by Student's *t*-test, and $P < 0.05$ was considered statistically significant.

Results

α-ESA induced apoptosis in HeLa cells

To investigate whether α-ESA has an anti-tumor activity, we examined cell viability in α-ESA treated cells using MTT assay. α-ESA significantly ($P < 0.05$–0.01) inhibited the growth of HeLa cells. Treatment with 100 μM α-ESA for 24 h resulted in about 40% growth inhibition (Fig. 1A) suggesting anti-tumor effect of α-ESA. Then, to examine whether α-ESA could induce apoptosis, we investigated by PI staining followed by examining the sub G1 phase in the cell cycle. HeLa cells treated with α-ESA showed dramatic increases in the sub G1 phase (Fig. 1B). These results indicate that α-ESA induces progress of apoptosis in HeLa cells. Taken together, these results suggest that α-ESA has capacity of anti-tumor effect together with ability of inducing apoptosis in HeLa cells.

α-ESA induced autophagy in HeLa cells

Next, to investigate the effect of α-ESA on autophagy formation, we observed autophagic vacuoles in α-ESA-treated cells through fluorescence microscopy after monodansylcadavarine (MDC), a specific marker for autophagic vacuoles [15], staining. α-ESA-treated cells showed typical autophagy formation when compared with control cells (Fig. 2A). To quantify the incidence of α-ESA-in-

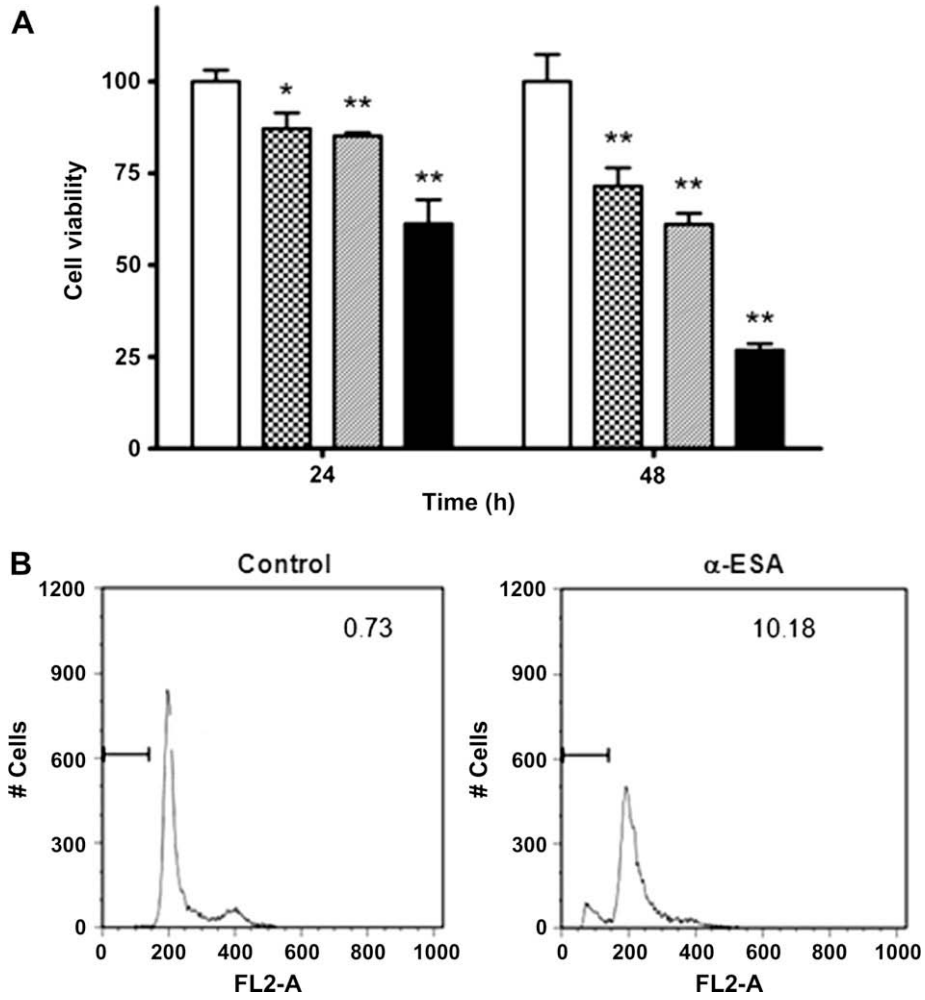

Fig. 1. α-ESA inhibits cell viability and induces apoptosis in HeLa cells. (A) HeLa cells were treated with 0, 25, 50 and 100 μM α-ESA for the indicated time periods, then an MTT assay was performed. Values are means ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control). Empty box, control; box with dot, 25 μM α-ESA; box with left slant lines, 50 μM α-ESA; filled box, 100 μM α-ESA. (B) HeLa cells were treated with 75 μM of α-ESA for 12 h, and the α-ESA-induced sub G1 area was analyzed by flow cytometry. The number in the upper right of each panel indicates the percentage of cells with apoptosis.

duced autophagy, we analyzed the MDC fluorescent intensity of α-ESA-treated cells using flow cytometry. α-ESA-treated cells induced significantly ($P < 0.05$) higher autophagy formation than did the control cells (Fig. 2B). The formation of autophagic vacuoles was further assessed and confirmed by transmission electron microscopic test. In contrast to the normal morphology of control cells, α-ESA-treated cells showed typical autophagous morphology, such as autophagic vacuoles in the cytoplasm (Fig. 2C). These results indicate that α-ESA induced autophagy formation. To identify whether the role of α-ESA-induced autophagy in HeLa cells is a protective or death promoting mechanism, we tested cell viability through MTT assay. For this, the cells were pretreated with 3-MA, an autophagy inhibitor, 1 h prior to administration of α-ESA. Viability of the cells treated with 3-MA prior to α-ESA treatment was significantly ($P < 0.05$) decreased compared to that of α-ESA-treated cells without 3-MA treatment (Fig. 2D). Taken together, these results suggest that α-ESA induced autophagy formation, which could play a pivotal role in cell survival.

α-ESA influenced the phosphorylation of AKT/mTOR and ERK1/2 and the conversion of LC3 I to LC3 II

Because the AKT/mTOR signaling pathway is known to be a main negative regulatory pathway in the inductions of apoptosis [6] and autophagy [16,17], we examined the effect of α-ESA on these signaling molecules using Western blot analysis. α-ESA decreased phosphorylation of P70S6K, downstream of mTOR, and AKT effectively in both a time- (Fig. 3A) and dose-dependent manner (Fig. 3B). It has been shown that the activation of the MAPK signaling pathway, which includes ERK1/2, is involved in the formation of autophagy [18]. Our results also coincide with this result that treatment with α-ESA increased phosphorylation of ERK1/2 in a time- (Fig. 3A) and dose-dependent manner (Fig. 3B) in HeLa cells. In addition, since it is known that a cytosolic protein called LC3 I is converted to the hallmark autophagosome-associating protein LC3 II [19], we examined the conversion of LC3 I to LC3 II in HeLa cells treated with α-ESA by Western blotting. The results showed that α-ESA treatment increased the conversion of LC3 I to LC3 II while controls decreased the conversion of LC3 I to LC3 II in a time- (Fig. 3A) and dose- (Fig. 3B) dependent manner. Taken together, these results demonstrate that α-ESA induced apoptosis and autophagy through direct inhibition of the phosphorylation of AKT/mTOR and activation of the phosphorylation of ERK1/2.

α-ESA treatment induced the ROS generation that impedes induction of apoptosis and formation of excessive autophagy

Conjugated fatty acids are known to be modulators of oxidative stress [1,2]. Recently, it has been reported that certain cellular stresses such as the presence of reactive oxygen species (ROS)

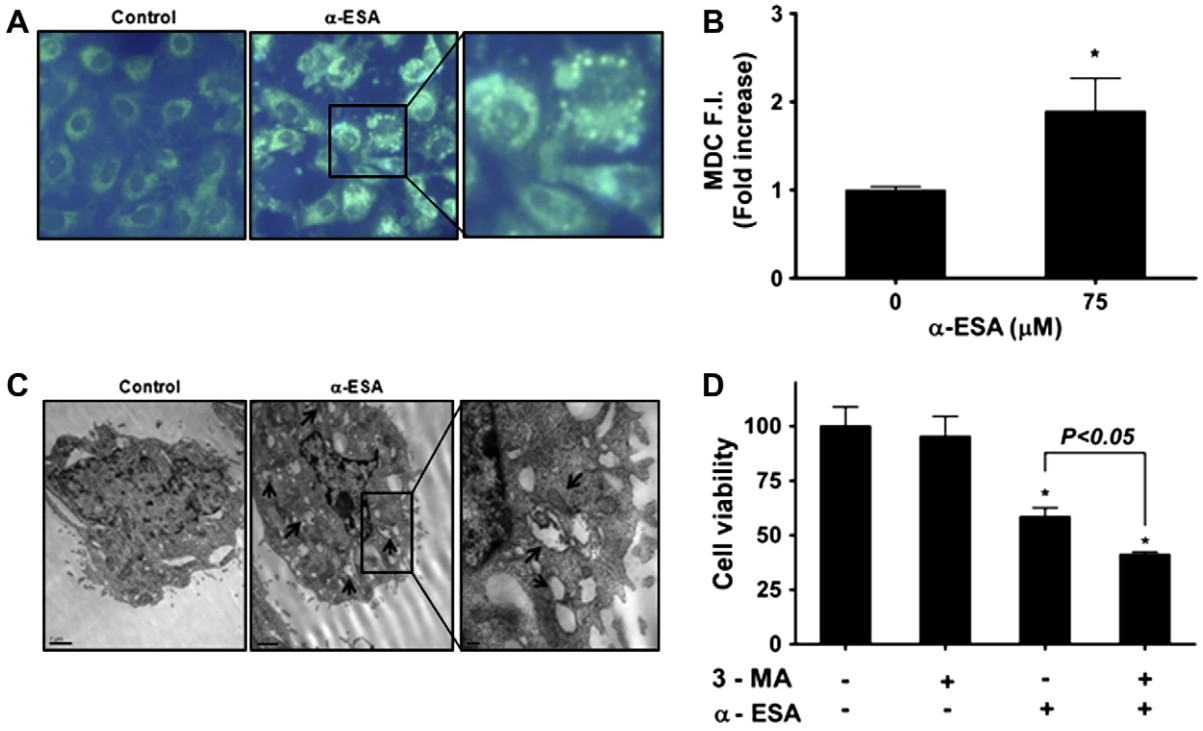

Fig. 2. α-ESA induces autophagy in HeLa cells. HeLa cells were treated with 75 μM α-ESA for 12 h and (A) the autophagosomes were observed under a fluorescence microscope (magnification; 400×) and (B) analyzed by flow cytometry after MDC staining. Values are means ± SD (*$P < 0.05$ *vs.* the control). (C) Autophagic vacuoles were observed using a transmission electron microscope. Arrows indicate autophagic vacuoles. Scale bar: 0.2 μm for first two panels and 1 μm for the third one. (D) HeLa cells were treated with 0.5 mM of 3-MA, an inhibitor of autophagy, 1 h prior to 75 μM of α-ESA treatment for 48 h, and cell viability was measured by using MTT assay. Values are means ± SD (*$P < 0.05$ *vs.* the control).

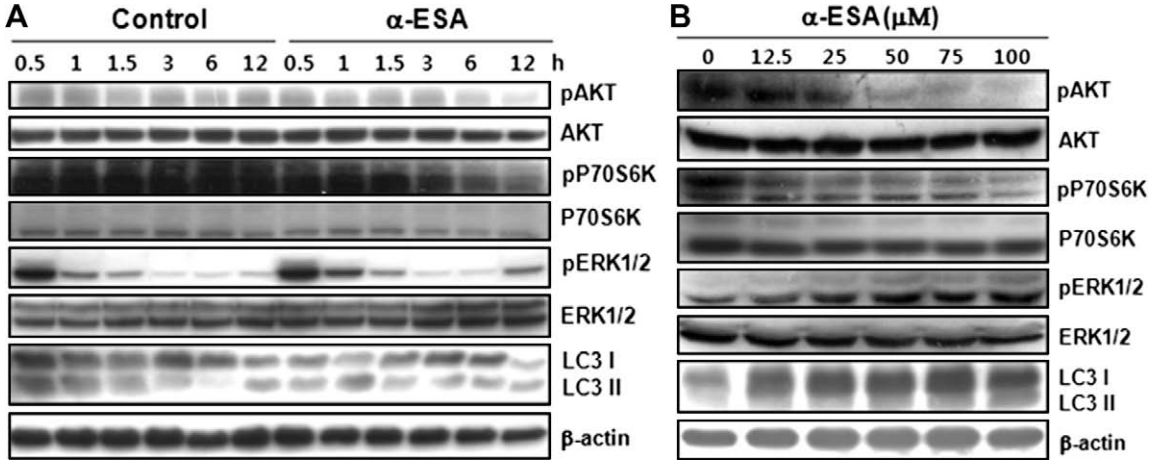

Fig. 3. α-ESA inhibits AKT/mTOR, activates ERK1/2 signaling and promotes conversion of LC3 I to LC3 II in HeLa cells. The cells were treated with (A) 75 μM α-ESA for the indicated time periods or (B) the indicated concentrations of α-ESA for 90 min for the detection of pAKT, pP70S6K, pERK1/2 and for 6 h for the detection of LC3. Changes in pAKT, pP70S6K, pERK1/2, and LC3 were examined by Western blot.

are involved in the regulation of apoptosis and autophagy formation [20,21]. To determine whether ROS were produced in α-ESA-treated HeLa cells, intracellular ROS levels were measured by flow cytometry after being labeled with CM-H_2DCF-DA (DCF), a specific ROS-detecting fluorescent dye. Exposure of HeLa cells to α-ESA for 2 h dramatically increased DCF fluorescence intensity (Fig. 4A). Furthermore, the cells pretreated with NAC, an ROS scavenger, 1 h prior to administration of α-ESA obviously decreased the fluorescence intensity of DCF compared to those of the α-ESA-treated cells without NAC pretreatment (Fig. 4A). It is evident that the α-ESA-induced ROS accumulation was also detected from 30 min up to 12 h in HeLa cells (data not shown). To examine the role of ROS in α-ESA-induced apoptosis and autophagy, we observed the phosphorylation of AKT, P70S6K and ERK1/2 and the conversion of LC3 I to LC3 II in α-ESA-treated HeLa cells after pretreatment with NAC. The phosphorylations of P70S6K and AKT were down-regulated in α-ESA-treated cells by pretreatment with NAC while the phosphorylation of ERK1/2 and conversion of LC3 I to LC3 II was enhanced when compared to that of the α-ESA-treated cells without NAC pretreatment (Fig. 4B). These results are consistent with blocking ROS accelerated α-ESA-induced apoptosis (Fig. 4C, upper panel) and autophagy (Fig. 4C, lower panel) as determined by flow cytometry with PI staining and MDC staining, respectively. Taken together, these results demonstrate that the generation of

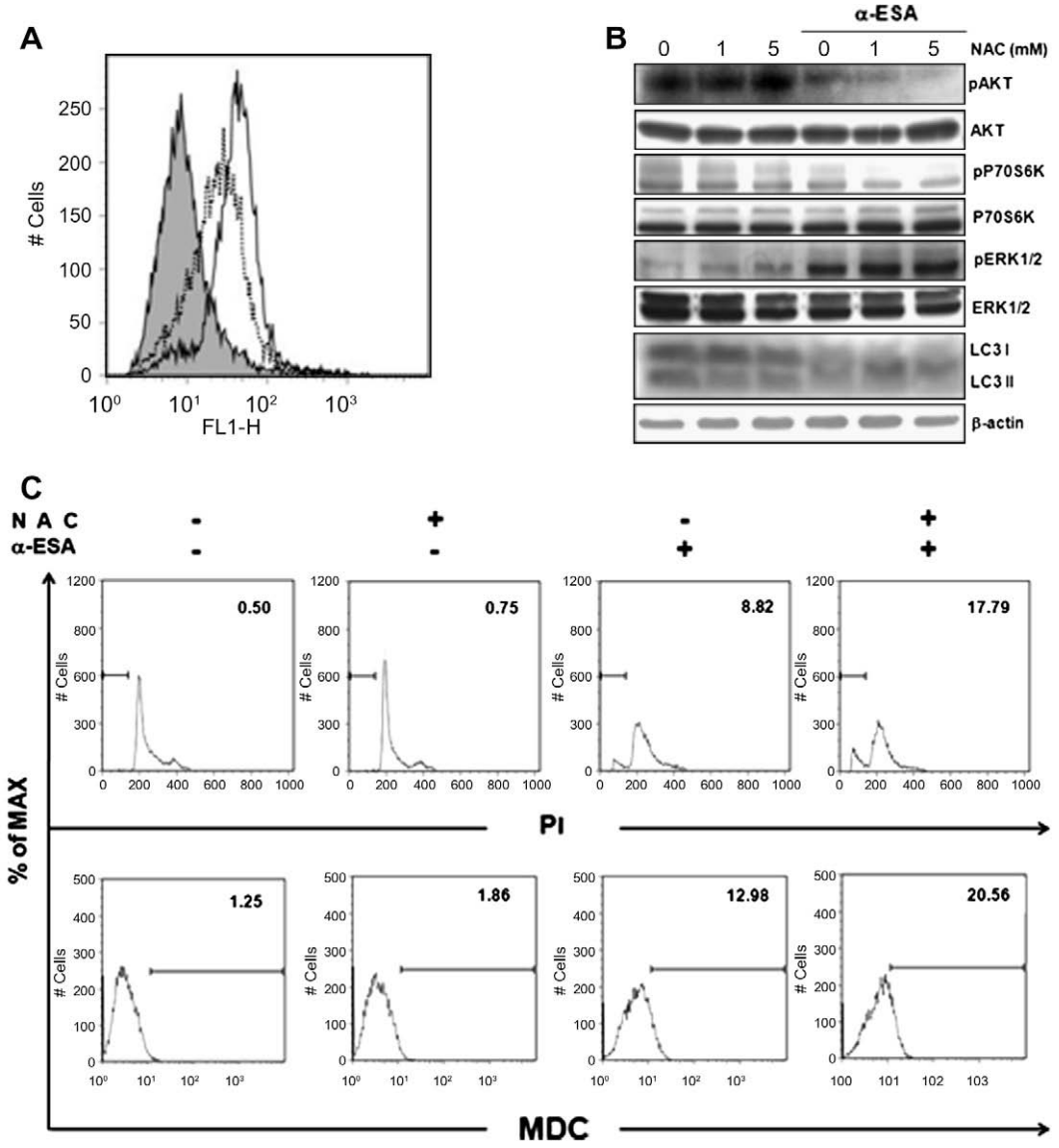

Fig. 4. ROS induced by α-ESA treatment impede the progress of apoptosis and formation of autophagy in HeLa cells. (A) The cells were treated with 5 mM NAC, an ROS scavenger, 1 h prior to the administration of 75 μM of α-ESA for 2 h. The cells were stained with CM-H_2DCF-DA and analyzed by flow cytometry. Gray area, control; dotted line, NAC and α-ESA; solid line, α-ESA. (B) The cells were incubated with 0, 1 or 5 mM NAC 1 h and treated with 75 μM α-ESA for 90 min for the detection of pAKT, pP70S6K, pERK1/2 and for 6 h for the conversion of LC3 I to LC3 II. Western blot experiments were performed on cell lysate with antibodies to pAKT, pP70S6K, pERK1/2 and LC3B. (C) The cells were treated with 5 mM NAC for 1 h prior to the 75 μM of α-ESA treatment for 12 h, and sub G1 phase in cell cycle (upper panel) and MDC fluorescence intensity (lower panel) was analyzed by flow cytometry. The number in the upper right of each panel indicates the percentage of cells with apoptosis (upper panel) and autophagy (lower panel), respectively.

ROS by α-ESA treatment impedes the progress of apoptosis and excessive autophagy formation which play a death-promoting role.

Discussion

Resistance to cell death is a characteristic hallmark of malignant tumor cells. Therefore, induction of cell death, such as apoptosis, in vitro and in patients with cancer has been a hot issue for physicians, biologists and clinicians in the fields of cancer cell biology and cancer therapy. Recently, effective induction of apoptosis in cancer cells through autophagy modulation has been suggested [22].

The aim of the present study was to identify the effect of α-ESA as an anti-cancer agent, especially focusing on the inter-relationship between apoptosis and autophagy formation. We have demonstrated that (i) α-ESA induces apoptosis and autophagy (ii) together with generation of ROS in HeLa cells. (iii) The autophagy formation induced by α-ESA treatment is a protective against cell death. (iv) ROS induced by α-ESA treatment impedes progress of apoptosis and (v) excessive autophagy formation leading to accelerate cell death.

It has been suggested that the AKT/mTOR signaling pathway negatively regulates progress of apoptosis [6] and autophagy formation [16,17], while the MAPK pathway, which includes ERK1/2, positively regulates autophagy formation [18]. In the present study we have clearly demonstrated that α-ESA inhibited AKT/mTOR signaling and activated ERK1/2 signaling, resulting in conversion of LC3 I to a hallmark of autophagy, LC3 II. In the context of induction of autophagy, Aoki et al. [23] and Ellington et al. [24] showed that natural products induce autophagy through the inhibition of AKT/mTOR signaling together with increased signaling in the ERK1/2 pathway. Although the results from other groups partially coincide with our results, the present findings are novel in light of the fact that α-ESA induced apoptosis and autophagy, and at the same time targeted AKT/mTOR and ERK1/2 signaling in HeLa

cells. Recently, as a potential therapeutic approach for malignant tumors, dual targeting of the AKT and mTOR signaling pathways has been suggested in the field of chemotherapy [25]. Hence, our results also agree with such consideration for the potential use of α-ESA as an anti-cancer agent.

The regulation of tumor cell death could be accomplished by a dynamic balance between apoptosis and autophagy [26], and the inter-relationship of these pathways seems to be complex. Accumulating data has revealed cases in which autophagy can be antagonistic or agonistic to the apoptotic pathway [27]. In the present study, we have demonstrated that α-ESA induced apoptosis and autophagy formation which resists cell death suggesting that the use of α-ESA together with autophagy inhibitor(s) may improve therapeutic strategies against cancer.

Our study also points out that ROS generated by α-ESA treatment impeded the progress of apoptosis and autophagy formation in HeLa cells. This was further proved by pretreatment with an ROS scavenger, which induced inactivation of pAKT and pP70S6K, activation of pERK1/2 and high amounts of LC3 II protein. When considering autophagy formation involved in cell survival in the present study, it would be expected that apoptosis further decreased by blocking of ROS in cells treated with α-ESA since autophagy formation is further increased in these cells. However, to our surprise, when α-ESA-induced ROS was blocked, apoptosis increased. Levine et al. reported that autophagy is a survival mechanism activated in response to nutrient deprivation; however, excessive autophagy would engage the cell death such as apoptosis and necrosis [28,29]. Taken together, our results suggest that excessive autophagy formation leads to cell death whereas α-ESA-induced ROS inhibit apoptosis and excessive autophagy formation in HeLa cells. Thus, we postulate that α-ESA-induced autophagy plays a pivotal role in cell survival through the generation of ROS which down-regulates excessive autophagy formation and therefore cell death.

Consequently, the use of α-ESA together with antioxidants may improve the therapeutic efficacy of inducing cancer cell death. However, the mechanism by which α-ESA induces ROS generation remains largely unknown. It has been reported that the accumulation of ROS may result from decreased degradation of ROS, either selective degradation of catalase [30] or inhibition of superoxide dismutase (SOD) [31]. Further study on the role of the catalase-SOD antioxidant system in α-ESA-induced ROS accumulation is needed.

In conclusion, our results show that α-ESA induces apoptosis and autophagy and acts concomitantly with ROS generation, impeding activation of key signaling molecules such as AKT/mTOR and ERK1/2 on the progress of apoptosis and excessive autophagy in HeLa cells.

Acknowledgments

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