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Inhibition of autophagy promoted sphingosylphosphorylcholine induced cell death in non-small cell lung cancer cells

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ABSTRACT

Sphingosylphosphorylcholine (SPC) is a bioactive lipid mediated popular cell apoptosis in cancer cells. As a cell-specific sphingolipid, its function in lung cancer cells is unknown. Here we showed that SPC treatment triggered necrosis and autophagy but inhibited apoptosis in two non-small cell lung cancer cell lines: A549 cell line and H157 cell line. Then 3-methyladenine (3-MA), an autophagy inhibitor, was introduced to clarify the relationships between autophagy and necrosis or apoptosis. 3MA suppressed the survival furtherly by promoting apoptosis while had no influence on necrosis. Subsequent studies revealed that activity of AKT and mammalian target of rapamycin (mTOR) complex 1 (mTORC1) were downregulated during autophagy. Furthermore, SPC failed to promote autophagy in p53 deleted cells. Thus SPC induced autophagy in non-small cell lung cancer cells was through AKT/mTORC1 and P53 signal pathway. Besides, SPC reduced both the mitochondria membrane potential and ROS level in A549 cells. These findings provided a molecular basis of SPC-stimulated A549 cell death and support the notion that inhibition of autophagy is likely a novel anticancer mechanism.

1. Introduction

Lung cancer is gaining more attention in recent years for the rapidly increased incidence and mortality. The major treatment for cancer therapy is still surgery combining radiation and chemotherapy [1]. However, there are quite a part of patients that are not suitable for surgery. And neither radiation nor chemotherapy is able to cure cancer effectively. Thus finding an effective biomarker for timely diagnosis of the malignant lung cancer is extremely urgent.

Sphingosylphosphorylcholine (SPC) is one of the members of sphingomyelin metabolites family. As is the case with ceramide, sphingosine-1-phosphate (S1P) or lysophosphatidic acid (LPA), SPC works not only as the membrane component, but also as a cell signal mediator. By employing of mass spectrometry, the levels of SPC in lots of pathological tissue such as atopic dermatitis patients [2], cerebrospinal fluid after subarachnoid hemorrhage [3], Niemann–Pick disease [4] and the malicious ascites of tumor patients [5] were determined to be elevated. These emphasized the physiological and pathological significance of SPC in corresponding diseases. Specifically, autotoxin (ATX), an enzyme that catalyzed sphingomyelin into SPC were detected significantly upregulated in ascites from ovarian cancer patients, which confirmed the pivotal role of SPC in tumor development [6]. SPC exhibited inhibitory effect on proliferation of most tumor cells such as pancreatic tumor cells [7], epithelial Ovarian Carcinoma (HEY) [8], mouse neuroblastoma cell lines [9] and so on. SPC was also reported to promote the migration of pancreatic cancer cells through MEK-ERK and Tgase-2-JNK signaling regulated keratin 8 phosphorylation and nuclear reorganization [10]. However, the influence of SPC on lung cancer cells has no report.

Autophagy exits in cells as a highly conserved event that clearing away those "old" proteins and impaired organelles to sustaining the cellular homeostasis. It can be stimulated under diverse stress conditions [11,12]. Lots of studies have concluded that autophagy played a critical role in cancer cell fate determination. Autophagy was capable of serving as either a pro-survival process or a cell death promoter [13–15]. And the cell death caused by autophagy was clarified as the autophagic cell death. Further investigation remained to be performed to clarifying the relationship between autophagy and cell death [16].

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2. Materials and methods

2.1. Reagents

SPC was purchased from Sigma (St. Louis, MO, USA) and dissolved in ethanol at 0.01 M as a stock solution. RPMI 1640 medium was purchased from Gibco (Grand Island, NY, USA). Calf serum was purchased from Hyclone Lab (Logan, UT). Dimethyl sulfoxide (DMSO), PepstatinA, and 3-methyladenine (3-MA) were from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cell culture and treatment

Human NSCLC cell lines A549 and H157 cells were cultured in RPMI 1640 supplemented with 10% calf serum and 150 μg/ml penicillin/streptomycin. The cultures were maintained at 37 °C in 5% CO_2 incubator. During the experiment, A549 and H157 cells were incubated in RPMI 1640 medium containing 1% calf serum in the presence of SPC or equal concentration of ethanol (control group). The cells cultured in RPMI 1640 medium supplemented with 10% calf serum were the normal control (normal group).

2.3. Morphology changes

Changes in the cellular morphology when challenged with indicated concentrations of SPC for 6 h were photographed using an inverted microscope (IX71, Olympus, Tokyo, Japan) at 200× magnification.

2.4. Cell viability assay

When the cells cultured on 96-well cell culture plate reached sub confluence, the cultures were changed with the RPIM 1640 containing 1% calf serum with indicated concentrations of SPC. After treatment for 12 h, cell growth was determined by SRB assay. The percentage of living cells was calculated by the ratio of optical density in the experimental wells to that in the normal wells.

2.5. LDH assay

When the cells cultured on 96-well cell culture plate reached sub confluence, the cultures were changed with the RPIM 1640 containing 1% serum with indicated concentrations of SPC. After treatment for 12 h, the LDH release was detected by LDH cytotoxicity assay kit (Cayman, Ann Arbor, MI, USA).

2.6. Western blot

After treatment with SPC for indicated times, A549 and H157 cells were lysed in lysis buffer containing 25 mM Tris–HCl (pH 6.8), 2% SDS, 6% glycerol, 1% 2-mercaptoethanol, 2 mM PMSF, 0.02% bromphenol blue and a protease inhibitor cocktail for 10 min at room temperature and boiled for another 10 min. Equal amounts of total proteins (30 μg) underwent 15% SDS PAGE and were electroblotted onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% (w/v) non fat dry milk in PBS-Tween 20 (PBST; 0.05%) for 1 h and incubated with primary antibody (1:1000 in PBST) at 4 °C overnight. After three washings in PBST, the PVDF membrane was incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h at room temperature. The immunoreactive bands were developed the ECL western blotting system. β-Actin was used as loading control. The relative quantity of proteins was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

2.7. Mitochondria membrane potential measurement

Mitochondrial membrane potential was estimated by JC-1 (Invitrogen, Carlsbad, California, USA) aggregates that are formed as a function of inner MMP. The formation of JC-1 aggregates and their fluorescence responds linearly to an increase in membrane potential. After treatment, the cells plated on 24-well plates were incubated in a humidified incubator at 37 °C with JC-1 4 μg/ml for 15 min. Then cells were washed twice with PBS and subjected to fluorescence (for red fluorescence, excitation, 543 nm; emission, 600 nm; for green fluorescence, excitation, 488 nm; emission, 535 nm) ratio detection. We randomly selected the region of interest (ROI) and then zoomed in on the same frames. The relative ratio of red/green fluorescence intensity values was used for data presentation.

2.8. ROS measurement

Intracellular ROS levels were measured with 2′, 7′-dichlorodihydrofluorescein (DCHF, Sigma, St. Louis, USA), which could be rapidly oxidized into the highly fluorescent 2′, 7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. The cells were treated with 10 μM SPC for 12 h, then washed cells with basal RPIM 1640 for 5 min and incubated the cells with DCHF 10 μM at 37 °C for 30 min. After washing the cells three times for 5 min with basal DMEM medium, the fluorescence was monitored with a confocal laser scanning microscope (Leica TCS SP2) using excitation and emission wavelengths of 488 and 535 nm, respectively. The amount of ROS was quantified as the relative fluorescence intensity of DCF per cell in the scan room by Leica Confocal Software (LCS Lite). The photographs were representatives of three independent experiments.

2.9. Statistical analysis

Data are from at least three independent experiments and expressed as mean ± standard error (SE). Statistical analysis involved the paired Student *t* test and ANOVA with SPSS version 11.5. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. SPC induced necrosis and inhibited apoptosis in non-small cell lung cancer cells

To know that the function of SPC is general for non-small cell lung cancer cells, we used two NSCLC cell lines, A549 and H157 cells. Cells were incubated with SPC for 12 h with indicated concentrations. The morphology changes and SRB assay showed that SPC inhibited A549 and H157 lung cancer cell growth at 5 μM and 10 μM (Fig. 1A, B and D). To determine which way was responsible for SPC-induced cell death, we detected the LDH release and the level of apoptosis related proteins. As a result, SPC promoted the release of cellular LDH (Fig. 1C and E) and inhibited the cleavage of PARP-1 (Fig. 1F), indicating that SPC induced necrosis and inhibited apoptosis in non-small cell lung cancer cells. Hence SPC induced cell loss was through necrosis but not apoptosis.

3.2. SPC induced autophagy in non-small cell lung cancer cells

Autophagy is a cellular process degrading longlived proteins and dysfunctional organelles. Our experiments showed that exposing A549 and H157 lung cancer cells to SPC induced the accumulation of LC3-II in a time (Fig. 2A) and dose (Fig. 2B) dependent

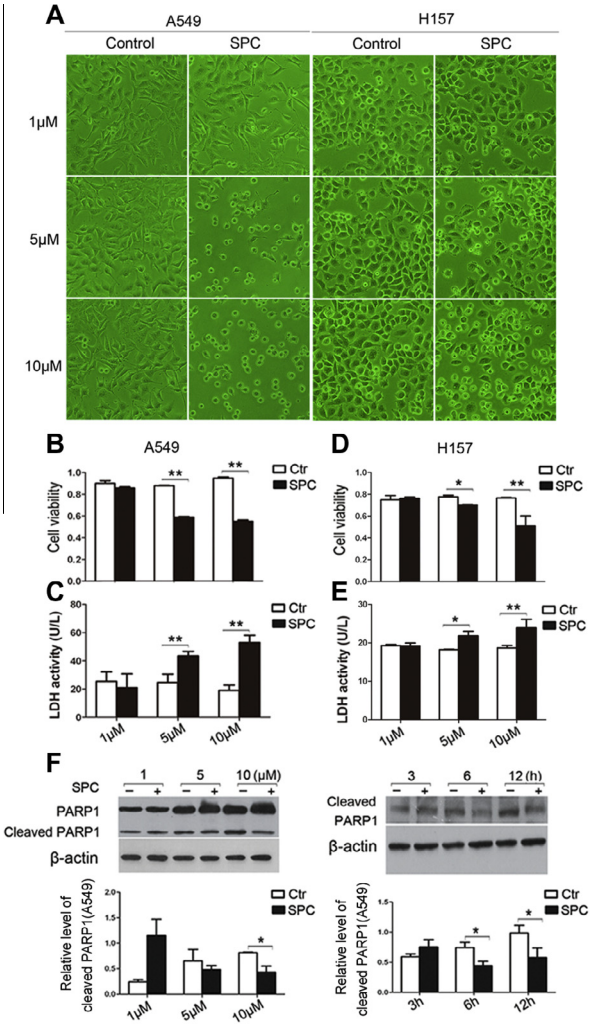

Fig. 1. Effect of SPC on cell growth of non-small cell lung cancer cells. The A549 and H157 cells were challenged with the indicated concentrations of SPC for 12 h. Cell morphology was observed with the phase-contrast microscopy (A). Cell viability and necrosis were analyzed by SRB assay (B, D) and LDH release (C, E). (F) A549 cells were challenged with the indicated concentrations of SPC for 12 h or treated with 10 μM SPC for 3, 6 and 12 h. Level of cleaved PARP1 was analyzed by Western blot. ($^{**}P < 0.01$; $^{*}P < 0.05$; $n = 3$).

manner. To verify the influence of SPC on the autophagy flow, we treated the cells with 3MA and pepstatin A before the addition of SPC. 3MA, an autophagy initiating inhibitor, suppressed SPC induced accumulation of LC3-II in these two cell lines (Fig. 2C). Pepstatin A, inhibiting the lysosome enzyme cathepstin D activity thereby blocking the autophagy flow, promoted the SPC induced accumulation of LC3-II in A549 cells (Fig. 2D). Results above concluded that SPC promoted the autophagy flux in non-small cell lung cancer cells.

Autophagy possessed the potential to induce non-apoptotic programmed cell death, which is clarified as type II programmed cell death [16]. Since cell loss induced by SPC was independent of apoptosis, we wondered whether it was caused by this autophagic cell death. We suppressed autophagy with 3MA to determine the role of autophagy in cell death. Cell death was not reduced but furtherly increased accompanied by the enhanced apoptosis (Fig. 2E and G), while SPC induced LDH release was not reverted with the treatment of 3MA (Fig. 2F). So we concluded that SPC induced autophagy only works as a negative regulator of apoptosis in non-small cell lung cancer cells.

3.3. SPC induced autophagy through AKT/mTOR pathway

There is convincing evidence that mTOR pathway represents the major regulatory mechanism of autophagy [17]. The activity of mTOR is primarily maintained by an active PI3K/Akt kinase pathway. Thus we detected whether this signal pathway mediated the autophagy triggered by SPC. Western blot analysis showed that 10 μM SPC effectively repressed the phosphorylation of p70S6K and 4EBP1 (two substrates of mTORC1) (Fig. 3A and B). AKT phosphorylation was inhibited by SPC at both 5 and 10 μM (Fig. 3A). These data proved that SPC induced autophagy through the AKT/mTORC1 signal pathway.

3.4. Involvement of P53 in SPC induced autophagy

According to our previous study, P53 was down regulated during the process that SPC promoted autophagy and inhibited apoptosis in human vascular endothelial cells (HUVECs) [18]. Thus we determined whether P53 participated in SPC induced autophagy in cancer cells by using two lung cancer cell lines: A549 (P53 wide type) and H1299 (P53 deficient type) cells. SPC induced accumulation of LC3-IIin P53 wide-type A549 cells (Fig. 4A). On the contrary, SPC did not induce accumulation of LC3-II in P53 deficient H1299 cells (Fig. 4A). These indicated that P53 may also participate in the regulation of SPC induced autophagy. We further detected the changes of mitochondria membrane potential and ROS levels in cells treated with SPC. SPC reduced the mitochondria membrane potential and ROS level (Fig. 4B and C).

4. Discussion

Ascites from patients with ovarian cancer showed markedly elevated levels of specific lysophospholipids as well as autotaxin, an enzyme involved in the production of SPC, suggesting a vital function of SPC in tumor progression [6]. SPC played a major role in the proliferation and/or migration of various cancer cells such as pancreatic cancer cells [7–10]. In this study, we further revealed that SPC induced abundant cell loss in two NSCLC cell lines: A549 and H157, which provided more evidence for SPC to be a melanogenic regulator. Thus targeting SPC can be developed as a potential new strategy for tumor therapy.

With the research of autophagy, the role of autophagy in cell death and the relationship between autophagy and apoptosis have been the focus of debate. According to our present results, autophagy induced by SPC functioned as an inhibiting factor of cell apoptosis in cancer cells. This strengthened the notion that autophagy was an anti-apoptosis mechanism of cancer cells. However, the overall functions of SPC in A549 and H157 cell lines had two sides. On the one hand, SPC promoted autophagy and inhibited apoptosis of the cells; on the other hand, SPC exhibited cytotoxicity through inducing necrosis. In HUVECs, SPC protected HUVECs deprived of serum and FGF from apoptosis by promoting autophagy [18]. Thus it implicated for us that SPC might be a popular autophagy inducer, but its effect was cell-type specific and dependent on relevant microenvironmental stimuli.

As a signal messenger, SPC was early reported to influence the intracellular Ca^{2+} level and activating the MAPK signal cascades, thus working as an effective mitogen for various cell type, specially for Swiss 3T3 fibroblasts [19]. Even though much of the function mechanisms of SPC have not been uncovered, it acted out great physiological and pathological effects in cardiovascular system, neuron system, the immune system and diverse cancer cells [20]. Our study showed that SPC was able to modulate the activity of AKT, and regulating mTORC1 which locates in the center of cell

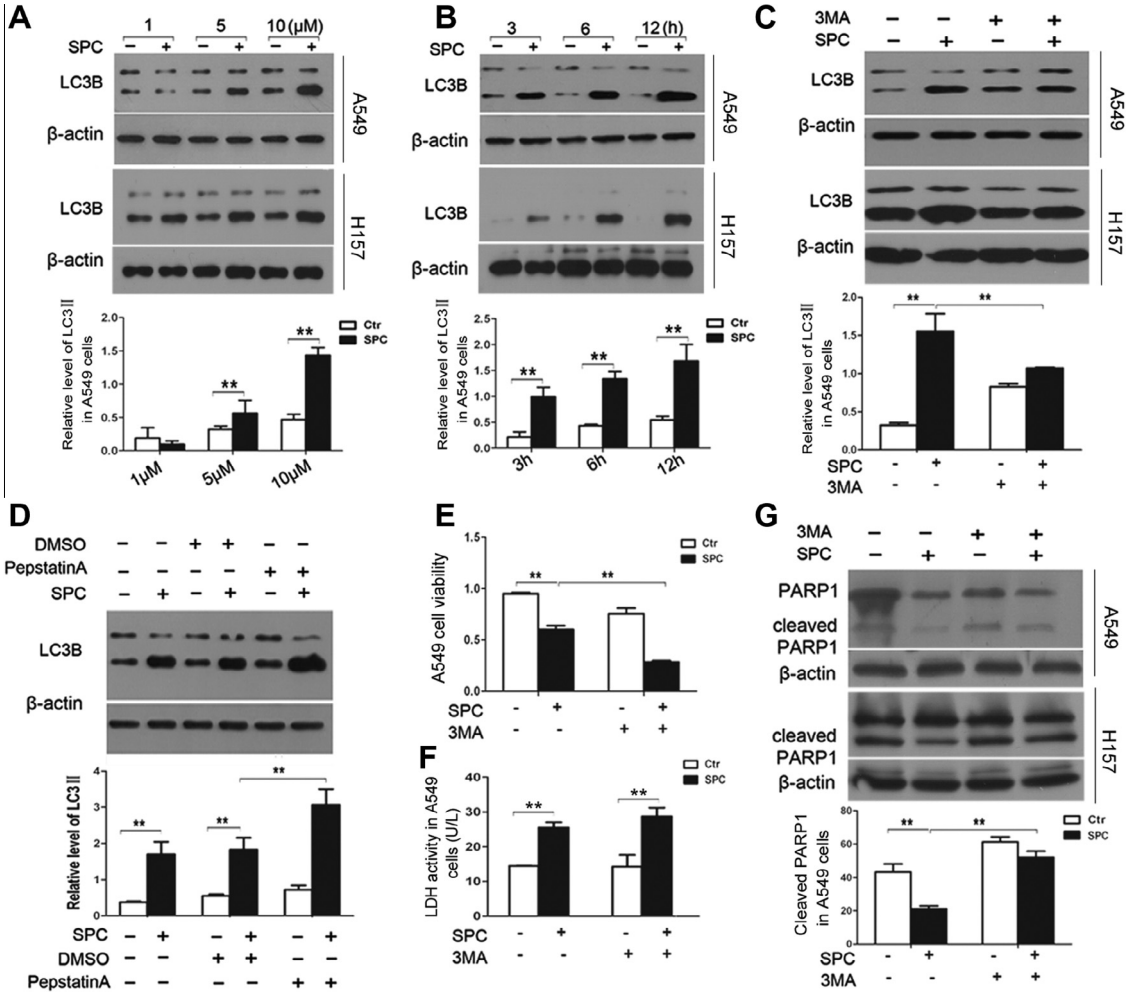

Fig. 2. Effect of SPC on autophagy in non-small cell lung cancer cells. (A) A549 and H157 cells were challenged with SPC of indicated concentrations for 6 h. Changes of LC3-II were observed by Western blot. (B) A549 and H157 cells were challenged with 10 μM SPC for the indicated times. Changes of LC3-II were observed by Western blot. (C) A549 and H157 cells were pretreated with 5 mM 3MA for 2 h before the addition of SPC (10 μM), and the LC3-II levels were detected by Western blot. (D) A549 Cells were pretreated with cathepsin D inhibitor pepstatin A (10 μM) for 4 h before the addition of SPC (10 μM), and the LC3-II levels were detected by Western blot. (E, F and G) Cells were pretreated with 5 mM 3MA for 2 h before incubating with SPC (10 μM) for 12 h. Cell viability and necrosis was analyzed by SRB assay (E) and LDH release (F), respectively. Cell lyses were subjected to Western blot for the detection of the cleaved PARP1 (G). ($^{**}P < 0.01$; $^{*}P < 0.05$; $n = 3$).

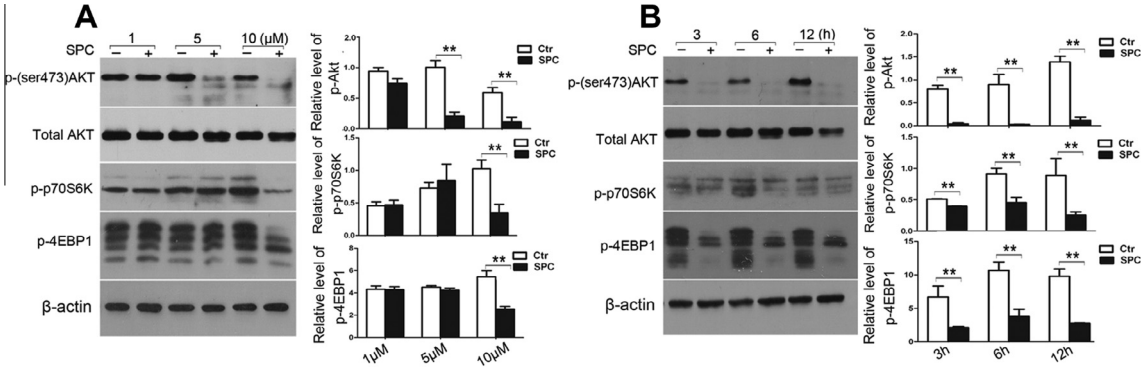

Fig. 3. Effect of SPC on AKT/mTORC1 signal pathway. (A) A549 cells were challenged with the indicated concentrations of SPC for 6 h, Western blot showed changes of phosphorylated AKT and two mTOR substrates: p70S6K and 4EBP1. (B) A549 cells were challenged with 10 μM SPC for the indicated times, Western blot showed changes of phosphorylated AKT and two mTOR substrates: p70S6K and 4EBP1. ($^{**}P < 0.01$; $^{*}P < 0.05$; $n = 3$).

metabolism. This allowed us to have a newer and deeper understanding of the function of SPC.

P53, a tumor suppressor gene, is a transcription factor which can control the expression of diverse apoptosis related genes and then affects the apoptosis signal pathway. P53 also participate in the manipulation of cell autophagy in transcription dependent and independent way [21]. Our previous study has identified SPC as a negative regulator of P53 cytosolic protein level during

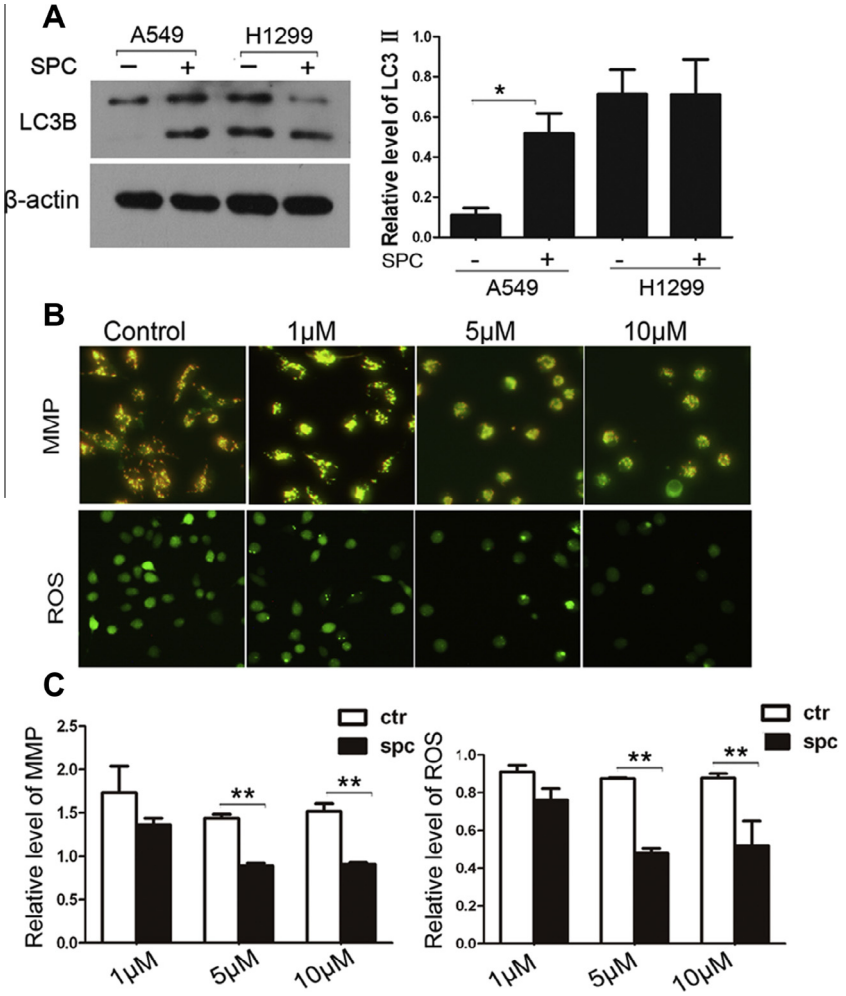

Fig. 4. Involvement of P53 in SPC induced autophagy. (A) P53 wide type non-small cell lung cancer (NSCLC) cell line A549 cells and P53 deficient cell line H1299 were challenged with 10 μM SPC for 12 h. Western blot showed the changes of LC3-II. (B, C) Cells were challenged with SPC of indicated concentrations for 12 h. The mitochondria membrane potential (MMP) and ROS levels were detected by use of JC-1 and DCFH probes, respectively. ($^{**}P < 0.01$; $^{*}P < 0.05$; $n = 3$).

autophagy in HUVECs [18]. SPC failed to induce autophagy in P53 deficient H1299 cells. This furtherly verified the participation of P53 in the process of SPC induced autophagy. The dissipation of the mitochondria membrane potential extensively served as the major characteristic of apoptosis and it was recently reported to be the inducer of mitophagy [22,23]. Oxidative stress caused by upward intracellular ROS also works as a moderator both in autophagy and apoptosis [24]. SPC can impact both these two indexes in A549 cells. Further investigation is required to be done to figure out the meanings of their changes in SPC induced autophagy.

In conclusion, our data showed that bioactive sphingolipid SPC regulated AKT/mTORC1 and P53 signal pathway in non-small cell lung cancer cells, thereby modulating autophagy and apoptosis of the cells. Inhibition of autophagy induced by SPC promoted cell loss through apoptosis. These results offered us new insight into the function of SPC in lung cancer cells and helped us to elucidating the relationship between autophagy and apoptosis.

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