

Autophagy inhibition through PI3K/Akt increases apoptosis by sodium selenite in NB4 cells

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Selenium possesses the chemotherapeutic feature by inducing apoptosis in cancer cell with trivial side effects on normal cells. However, the mechanism in which is not clearly understood. Emerging evidence indicates the overlaps between the autophagy and the apoptosis. In this study, we have investigated the role of autophagy in selenium-induced apoptosis in NB4 cells. We find that autophagy is suppressed in NB4 cells treated by sodium selenite, as measured by electron microscope, acridine orange staining and western blot. Moreover, selenite combined with autophagy inhibitor contributes to the up-regulation of apoptosis, while the PI3K/Akt signaling pathway is down-regulated. Consistently, when the inhibitor of PI3K was applied, the autophagic level significantly decreased. In summary, sodium selenite increases NB4 cell apoptosis by autophagy inhibition through PI3K/Akt, and the inhibition of autophagy contributes to the up-regulation of apoptosis. [BMB reports 2009; 42(9): 599-604]

INTRODUCTION

Autophagy is a regulated process that degrades and recycles cellular constituents, where parts of the cytoplasm or entire organelles are sequestered into double-membraned vesicles termed autophagic vacuoles or autophagosomes. These autophagosomes then fuse with lysosomes to mature into single-membraned autophagolysosomes, in which sequestered cytoplasmic components are degraded by lysosomal hydrolases (1, 2).

Although autophagy can serve as a protective mechanism against apoptosis and starvation by recycling macromolecules and removing damaged mitochondria and other organelles, excessive autophagy results in cell death with appearance of excessive autophagic vesicles (3, 4). While apoptosis is called type I programmed cell death, autophagic cell death is named as type II programmed cell death. These two kinds of cell death are distinct from each other. However, more and more

evidence shows that there is a cross-talk between them (5). Recent studies indicate that the specific function of autophagy depends on the certain circumstances, including cell types, cellular context, and the nature of treatment.

Selenium is an essential trace element with the potential chemopreventive effects against various cancers (6-9). Our previous work has shown that 20 μ M sodium selenite can induce apoptosis in NB4 cells (10). However, whether autophagy is involved in selenium-induced apoptosis of NB4 cells remains unknown. Therefore, it is of potential clinical significance to better understand the molecular mechanisms regulating the autophagic pathway in selenium-induced apoptosis in NB4 cells.

The serine/threonine kinase, Akt (protein kinase B), is a downstream effector of PI3K. The activation of this pathway allows cells to inhibit apoptotic and autophagic programmed cell death, which may contribute to malignant transformation and tumor growth (11-13). Moreover, activated Akt also stimulates the mTOR/P70S6-kinase pathway, whose activation is required for the initiation of protein synthesis (14, 15). Our former studies showed that the expression of p-Akt, downstream factor of PI3K, decreased in selenium induced apoptosis in NB4 cells (16). Consequently, we postulated that the PI3K pathway plays an important role in the inhibition of autophagy and the increase of apoptosis in NB4 cells.

In this study, we find that the PI3K-Akt pathway promotes autophagy in NB4 cells. And Autophagy inhibitors enhance the apoptosis ratio in NB4 cells treated by sodium selenite.

RESULTS

Selenite increases apoptosis with inhibiting autophagy in NB4 cells

Autophagy is inhibited in selenium-induced apoptosis in NB4 cells as measured by electron microscopy (EM), acridine orange (AO) staining and western blot.

Autophagy is featured by the formations of autophagosome and autolysosome. Using EM, we identified that the number of double-membraned autophagosomes (indicated by arrows) decreased while empty vesicles increased after the treatment with selenium in NB4 cells (Fig. 1A). It indicates less autophagosome production with the inhibition of autophagy in a selenium induced time-dependent manner.

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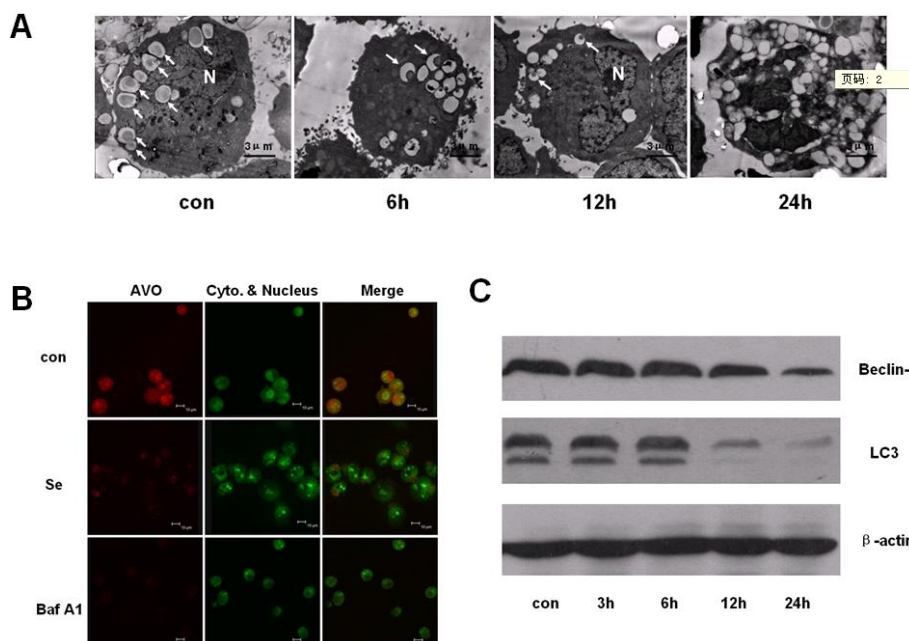


Fig. 1. Selenite inhibits autophagy in NB4 cells. (A) Electron microscopy pictures were taken of NB4 cells untreated (control) or treated with 20 μM selenite for 6 h, 12 h, 24 h respectively. Autophagosomes (arrow) and nucleus (N) are indicated. Scale bars, 3 μm . (B) After treatment with 20 μM selenite for 12 h, NB4 cells were stained with AO as described in the Material and Methods and detected by laser scanning confocal microscope. Non-treatment NB4 cells served as the positive control, with 100 nM Baf A1 treatment NB4 cells as the negative one. (C) After exposure to 20 μM selenite for 3 h, 6 h, 12 h, 24 h, NB4 cells were harvested and analyzed by Western blot with antibodies against Beclin-1 and LC3.

AO is a fluorescent weak base that accumulates in acidic spaces, such as autolysosomes and lysosomes, which are called acid vesicular organelles AVO fluoresce bright red, whereas the cytoplasm and nucleolus fluoresce bright green and dim red. After the culture of NB4 cells with or without selenite, cells were incubated with AO. And using cells incubated with Bafilomycin A1, an autophagy inhibitor, as negative control. Using Laser scanning confocal microscope, we found that bright red dots faded in NB4 cells after the treatment of selenite, while the green areas maintained (Fig. 1B).

LC3 exists in two forms, LC3-I and its proteolytic derivative of LC3-II (18 and 16 kDa, respectively), which are localized in the cytoplasm (LC3-I) or autophagosomal membranes (LC3-II). LC3-II thus can be used to estimate the abundance of autophagosomes before they are destroyed through the fusion with lysosomes. The amount of LC3-II is closely related with the number of autophagosomes and serves as a good indicator of autophagosome formation. As shown by the western blots in Fig. 1C, both LC3-I and II decrease in a time dependent manner in NB4 cells after the treatment of selenite.

Beclin-1 expression is shown to be involved in the formation of preautophagosomal structures. Similar to LC3-II, the expression of beclin-1 decreased following the treatment by selenite in NB4 cells (Fig. 1C).

Taken together, selenite induced apoptosis with inhibition of autophagy in NB4 cells.

Inhibition of autophagy increases selenite-induced apoptosis

Selenite induces apoptosis with inhibition of autophagy in NB4 cells. Therefore, we asked whether autophagy inhibition can increase the rate of apoptosis. We used two autophagy in-

hibitors to pre-treat NB4 cells for 1.5 h, and then sodium selenite was added into the system.

3-Methyladenine (3-MA) is an agent long known to selectively and potently inhibit autophagy-dependent protein degradation and suppress the formation of autophagosomes (17, 18), whereas bafilomycin A1 (Baf A1) appears to block the fusion of autophagosomes and lysosomes (19).

Both 3-MA and Baf A1 could inhibit autophagy at dose of 10 mM and 100 nM respectively in NB4 cells (Fig. 2A). Expressions of LC3 and Beclin 1 both decreased when NB4 cells treated with 3-MA. In Baf A1 treated cells, autophagy is inhibited before the fusion of autophagosomes and lysosomes. As a result, LC3-II aggregates on the autophagosomes, and can not be degraded through the fusion of autophagosomes and lysosomes. Therefore, in these cells, the increase of LC3-II expression indicates autophagy is suppressed.

After confirmed autophagy inhibitors can efficiently suppress autophagy in NB4 cells, we asked their effects on apoptosis in NB4 cells. We assessed the effects of autophagy inhibitors on caspases activation in NB4 cells by western blot. The results showed that the activated cleavage fragment of caspase-9 increased after exposure to autophagy inhibitors and combination treatment. In parallel with the activation of caspase-9, there were also increases in the cleavage of effector caspases, namely caspase-3 and -7 (Fig. 2B). The activation of caspase-9 indicated that mitochondria mediated apoptotic pathway might be involved in autophagy inhibitor induced cell apoptosis in NB4 cells. And Flow cytometry result is consistent to this. When NB4 cells were treated with 3-MA or Baf A1 combined with selenite, apoptosis ratio was significantly enhanced compared with the selenite alone treatment group

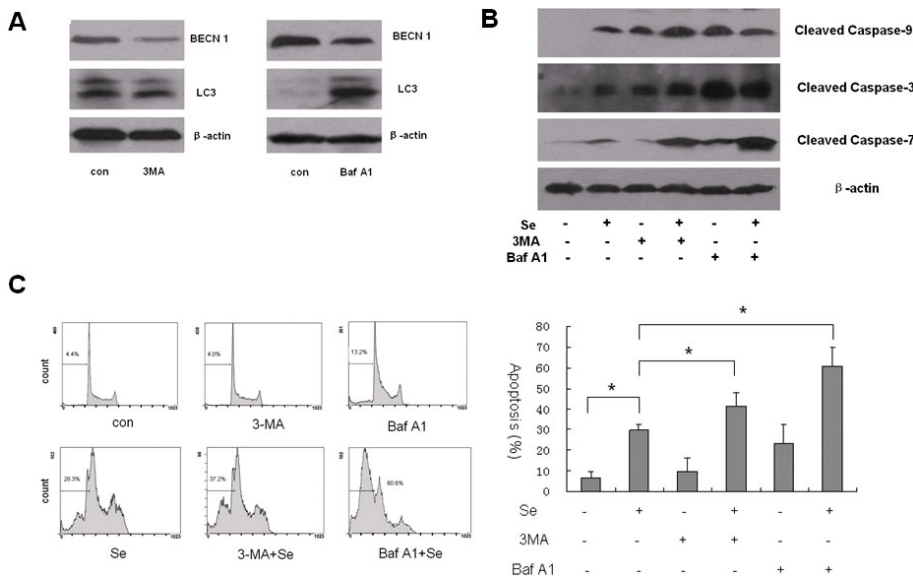


Fig. 2. inhibition of autophagy by 3-MA and Baf A1 increases selenite-induced apoptosis in NB4 cells. Cells were pre-treated with 10 mM 3-MA or 100 nM Baf A1 for 1.5 h prior to the selenium treatment for 24 h. (A) Western-blot analysis of the effect of autophagy inhibition by 3-MA or Baf A1, using antibodies against Beclin1 and LC3. (B) Western-blot analysis of caspase cleavage treated by selenium, 3-MA or Baf A1, using antibodies against cleaved caspase-9, cleaved caspase-3 and cleaved caspase-7. (C) Flow cytometry analysis of the effect of autophagy inhibitor 3-MA and Baf A1 on selenium-induced cell apoptosis. Data are presented as the mean ± SD of triplicates. *P < 0.05.

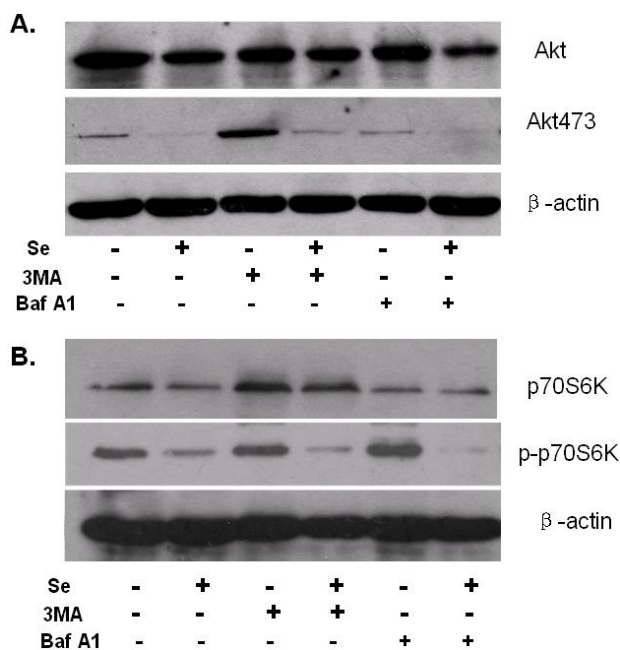


Fig. 3. Suppression of p-Akt and mTOR by autophagy inhibitor is enhanced with selenium. Cells were pretreated with 10 mM 3-MA or 100 nM Baf A1 for 1.5 h prior to selenium treatment for 24 h. (A) Western-blot analysis of the effect regarding p-Akt inhibition by selenium and its cotreatment with 3-MA and Baf A1. (B) Western-blot analysis of the effect regarding mTOR inhibition by selenium and its cotreatment with 3-MA and Baf A1.

(Fig. 2C). And Baf A1 alone can efficiently trigger apoptosis. Taken together, Inhibiting autophagy by 3-MA or Baf A1 can facilitate selenium-induced apoptosis in NB4 cells.

PI3K/Akt/mTOR pathway promotes autophagy

We already had known that autophagy inhibition by 3MA and BafA1 can enhance the selenium induced NB4 cells apoptosis. We further explored the mechanism behind this phenomenon.

Previous studies showed that p-Akt decreased when NB4 cells were treated with sodium selenite. Additionally, accumulating evidence supports that PI3K/Akt/mTOR signaling pathway is involved in autophagy (20-24). We investigated how this pathway functioned in inhibiting autophagy and promoting the apoptosis by selenium in NB4 cells. As shown in Fig. 3A, Akt phosphorylation decreased sharply when treated by selenium with or without autophagy inhibitor. Selenium could also inhibit p-p70S6K, the down effector of mTOR, which is regulated by p-Akt (Fig. 3B). Moreover, the autophagy inhibitors enhance the suppression extent by selenium. It indicates that the suppression of PI3K/Akt/mTOR signaling pathway is involved in autophagy inhibition and promotes apoptosis in NB4 cells treated by selenium.

LY294002 inhibits autophagy through inhibiting Akt

To further discuss whether Akt-inhibition contributes to the suppression of autophagy, the inhibitor of Akt, LY294002 (LY) was added into this system. EM results show that the formation of autophagosome decreased sharply after treated by LY, compared to control group. (Fig. 4B) In addition, Beclin 1 and LC3 also decrease in NB4 cells treated with LY. It indicates that the Akt inhibition could suppress autophagy (Fig. 4A).

DISCUSSION

As selenium is a promising clinical medicine in dealing with cancer, this study did further investigations regarding the mechanism of selenium induced apoptosis in NB4 cells.

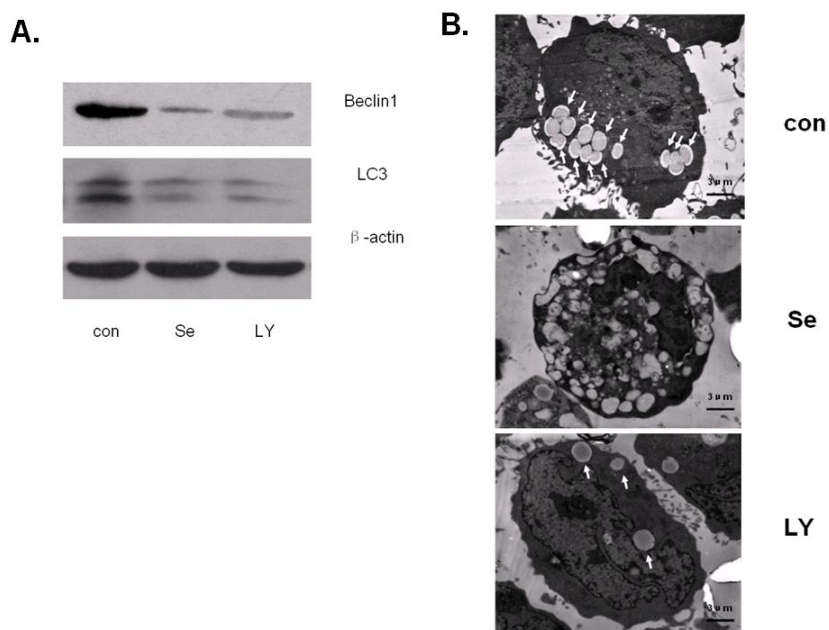


Fig. 4. Akt inhibitor suppressed autophagy and promoted selenite-induced apoptosis. Cells were treated with 20 μ M selenite and 20 μ M LY294002 for 24 h respectively. (A) Western-blot analysis of BECN1 and LC3 expression inhibited by LY294002. (B) Electron microscopy pictures were taken of NB4 cells untreated (control) or treated with 20 μ M LY294002 for 12 h respectively. Autophagosomes (arrow) and nucleus (N) are indicated. Scale bars, 3 μ M.

Autophagy is suppressed in NB4 cells treated by sodium selenite in a time-dependent manner. And combination treatment of autophagy inhibitors and selenium enhances the apoptosis ratio in NB4 cells. In addition, PI3K/Akt signaling pathway suppression contributes to the inhibition of autophagy in NB4 cells. These studies expand our understanding concerning the roles of autophagy in regulating apoptosis and provide important information of the molecular mechanisms of autophagy after selenium treatment in NB4 cells.

It was previously suggested that autophagy and apoptosis were distinct forms of cell death, but more and more recent data implies that there is a mechanistic overlap between these two types of cell death (3). Some observations indicate that autophagy plays a role in preventing cells from apoptosis through the sequestration of cytochrome c (25). Other evidence showed that active autophagy appeared to increase the tendency to undergo apoptosis (20, 26, 27). In our study, autophagy decreased in selenite-induced apoptosis in NB4 cells. Moreover, when autophagy was suppressed by 3-MA and Baf A1 in selenite-treated NB4 cells, apoptosis ratio was significantly enhanced compared to the selenite alone treatment group, suggesting autophagy could protect NB4 cells from death through antagonizing apoptosis induced by selenite. Autophagy serves a protective role in NB4 cells.

The function of PI3K-Akt pathway and its links to autophagy and apoptosis are disputing problems. Some reports described that PI3K-Akt activation suppresses autophagy in mammalian cells (21-24). However, emerging studies have pointed out that PI3K-Akt pathway positively regulates autophagy (20). In this study, PI3K-Akt was activated in an autophagic process, but suppressed in apoptosis. Many proteins, including PI3K, Akt,

and mTOR, are related to both apoptosis and autophagy. However, the specific associations among these proteins are still not clear. As a result, it needs to be studied in the future of whether continuous expression of p-Akt could inhibit apoptosis in NB4 cells. In summary, PI3K/Akt signaling is augmented in autophagy, while it is suppressed in apoptosis.

In conclusion, our results demonstrate that PI3K-Akt pathway promotes autophagy in NB4 cells. Autophagy facilitates the survival of NB4 cells. And the combined treatment of autophagy inhibitors and selenium raises the apoptosis ratio in NB4 cells than selenium treated alone.

MATERIALS AND METHODS

Reagents and antibodies

Sodium selenite, 3-Methyladenine, Bafilomycin and anti- β -actin were purchased from Sigma-Aldrich. Anti Beclin-1, LC3, Akt, phospho-Akt (Ser473), p70 S6 kinase, phospho-p70 S6 kinase (Thr389), cleaved caspase-9, cleaved caspase-3, cleaved caspase-7 were purchased from Cell Signaling Technology.

Cell culture

NB4 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Cell lysis and western blot analysis

Approximately 1×10^7 Cells were collected, washed twice with ice-cold phosphate buffered saline (PBS), and lysed in Cell lysis RIPA Buffer for 5 min on ice and then subjected to

sonication for 20 s. The lysate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was collected, and protein concentration was determined by the Bradford assay. Equal amounts of protein were separated by 15% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then blocked with Tris buffered saline-Tween-20 (TBST) containing 5% non-fat milk and incubated by primary antibodies overnight at 4°C. After washing with TBST, membranes were incubated by secondary antibodies conjugated with HRP for 1 h at room temperature. After a second round of washing with TBST, the blots were probed with the ECL system.

Flow cytometry analysis of apoptosis

Cells were washed twice with ice-cold PBS, and fixed with 70% ethanol at 4°C overnight. After washing with PBS, cells were incubated in 0.5 ml PBS containing 50 µg/ml RNase A for 30 min at 37°C, and then added PI to achieve the final concentration of 50 µg/ml for 30 min on ice in the dark. The resultant cell suspension was then subjected to flow cytometry analysis (COULTER EPICS XL).

Detection of acidic vesicular organelles with acridine orange staining

To detect acidic vesicular organelles (AVO), treated cells were stained with 1 µM acridine orange for 15 min. Cell images were captured with laser scanning confocal microscope (LEICA TCS SP2 SE) excitation wavelength: 488 nm; emission wavelength: green light, 530 nm. Red light, 640 nm).

Electron microscopy

NB4 Cells were collected and fixed in 2.5% glutaraldehyde for at least 3 h. Then cells were treated with 2% paraformaldehyde at room temperature for 60 min, 0.1% glutaraldehyde in 0.1 M sodium cacodylate for 2 h, postfixed with 1% OsO₄ for 1.5 h, after second washing, dehydrated with graded acetone, and was embedded in Quetol 812. Ultrathin sections were observed using a HITACHI H7100 electron microscope.

Statistical methods

All data and results presented were confirmed in at least three independent experiments. The data are expressed as means ± S.D with the statistical method of Student's t test. P < 0.05 was considered statistical significance.

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