

The prosurvival role of autophagy in resveratrol-induced cytotoxicity in GH3 cells

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Abstract. In a previous study, we reported that resveratrol exerts antitumor effects through the estrogen receptor in prolactinoma. The autophagy/lysosomal degradation pathway plays an important role in damage control and energy efficiency. In this study, we investigated the involvement of autophagy and the related signaling pathways in resveratrol-induced apoptosis of GH3 cells. We demonstrate that resveratrol inhibits cell proliferation and induces apoptosis in a dose-dependent manner in GH3 cells. The cleavage of PARP was also observed, and the activation of caspase-3 and caspase-8 was detected. Consistent with this finding, the inhibition of caspase activation effectively attenuated resveratrol-induced cell apoptosis. In addition, the decreased level of Bcl-2 was also observed. The induction of autophagy was confirmed by the detection of the formation of autophagic vacuoles, and the increase in microtubule-associated protein 1 light chain 3 (LC3)-II and beclin-1 levels, two hallmarks of autophagy. Pre-treatment with bafilomycin A1 or 3-methyladenine, inhibitors of autophagy, enhanced the resveratrol-mediated caspase activation and cell death. Moreover, resveratrol induced the activation of ERK1/2, as well as the downregulation of Akt and mTOR phosphorylation. Taken together, these findings indicate that resveratrol induces caspase-dependent apoptosis and decreases Bcl-2 levels. In addition, resveratrol-induced autophagy is regulated by the PI3K/Akt/mTOR and ERK1/2 pathways. Furthermore, the inhibition of autophagy increases the cytotoxicity of resveratrol to GH3 cells.

Introduction

It has been reported that resveratrol (trans-3,4',5-trihydroxy-stilbene), a phytoestrogen which is found in grape skin and red wine, has numerous beneficial effects, including the inhibition of tumor growth (1). It affects tumor initiation, promotion and progression through a wide range of signaling pathways (2,3). We recently observed that resveratrol inhibited the growth of prolactinoma and decreased the level of prolactin through the estrogen receptor (4). Estrogen plays an important role in prolactinoma (5), as well as in breast cancer; therefore, prolactinoma is an estrogen-dependent tumor (6,7). However, some antagonists of estrogen, such as tamoxifen, cannot exert satisfactory antitumor effects in prolactinoma as they have been shown to exert in breast cancer (8). The mechanisms of anti-estrogen resistance are still poorly understood in prolactinoma.

Autophagy is a mechanism by which a cell digests its own damaged subcellular organelles or unfolded/misfolded/aggregated proteins, in an attempt to maintain/restore homeostasis (9). Autophagy has been implicated in a variety of diseases, including neurodegeneration, aging, infection, myopathy and tumors (10). However, the role of autophagy in tumors is quite complex and remains somewhat controversial (11,12). Autophagy appears to play a suppressive role during tumor development, but contributes to tumor cell survival during cancer progression (13). Furthermore, tumor cells can use autophagy to resist various antitumor therapies (14,15). Certain studies have demonstrated autophagy can potentiate the resensitization of previously anti-estrogen-resistant breast cancer cells, indicating a close link between autophagy and antiestrogen resistance (16-18). However, the role of autophagy in prolactinoma has not yet been clearly elucidated.

The aim of the present study was to determine the effects of resveratrol and shed further insight into the crosstalk between autophagy and apoptosis in resveratrol-induced cytotoxicity in prolactinoma.

Materials and methods

Reagents and chemicals. Reagents and chemical sources were obtained from the following manufacturers: resveratrol was

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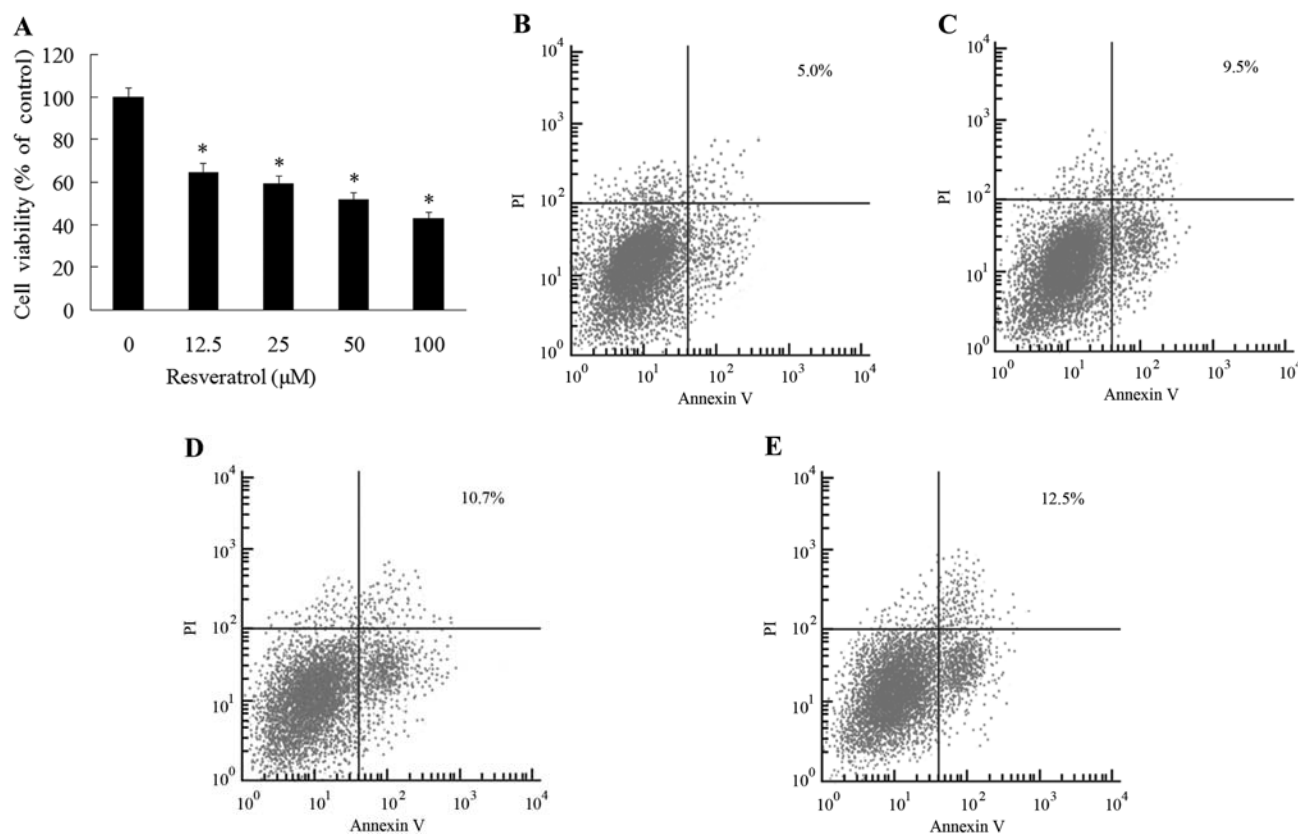


Figure 1. Resveratrol inhibits the viability and induces the apoptosis of GH3 cells. GH3 cells were treated with the indicated concentrations of resveratrol for 48 h. (A) Cell viability was determined by MTT assay. * $p < 0.05$, resveratrol vs. control ($n = 3$). (B-E) Cells were subjected to quantitative analysis of cell apoptosis by Annexin V and PI double staining flow cytometry. (B) Control (vehicle-treated) group. (C) Cells treated with 12.5 μM resveratrol. (D) Cells treated with 25 μM resveratrol. (E) Cells treated with 50 μM resveratrol.

purchased from Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA; Ham's F-10 medium was from Gibco-BRL, Carlsbad, CA, USA; fetal bovine serum was obtained from HyClone, Logan UT, USA; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-methyladenine (3-MA) were obtained from Sigma Chemical Co., St. Louis, MO, USA; specific inhibitors for caspase-3 (z-DEVE-fmk), caspase-8 (z-IETD-fmk) and pan-caspase inhibitor (z-VAD-fmk) were from BioVision, Mountain View, CA, USA; antibodies against Bcl-2, caspase-3 and caspase-8 were from Sigma Chemical Co.; antibodies against PARP, microtubule-associated protein 1 light chain 3 (LC3), beclin-1, phospho-mTOR and mTOR, and donkey anti-goat immunoglobulin G-fluorescein isothiocyanate were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; antibodies against PI3K, Akt, phospho-Akt and p38 were from BD Biosciences, Franklin Lakes, NJ, USA; and antibodies against ERK1/2, phospho-ERK1/2, phospho-p38 and phospho-JNK were purchased from Millipore Corp., Billerica, MA, USA.

Cell culture. GH3 cells are an established estrogen-responsive cell line from rat pituitary tumor cells, which can secrete prolactin. Therefore, they are a useful model for studying the effects of resveratrol on prolactinoma. GH3 cells were maintained in Ham's F-10 medium containing 12.5% horse serum, 2.5% fetal bovine serum, 2 mmol/L L-glutamine, 0.25 μg/ml fungizone and 80 μg/ml gentamycin. The cells were plated at various densities according to purpose and were incubated for 4 days in main-

tenance medium. Before treatment, the medium was replaced with experimental medium, which was a defined serum-free, phenol red-free medium containing Ham's F-12 medium with 10 μg/ml insulin, 5 μg/ml transferrin and 0.5 ng/ml parathyroid hormone for 24 h. As a negative control, cells were cultured in medium with serum without treatment; as a positive control, cells were cultured in serum-free medium without treatment; and the cells in the treatment group were cultured in serum-free medium with 50 μM resveratrol. All cells were cultured in a humidified chamber with 5% CO₂ at 37°C.

Cell viability assays. At the end of the incubation period, cell viability was assessed by MTT assay. Briefly, 125 μg/well of MTT were added to the treatment wells, and 2 h later, 100 ml of developer solution [50% v/v dimethylformamide (DMF), 20% w/v sodium dodecyl sulfate (SDS), 0.24% v/v glacial acetic acid and 60 mM sodium acetate] were added. The optical density at 570 nm was determined. Data are presented as optical density or as a percentage of the control.

Analysis of apoptosis. GH3 cells were treated with the vehicle (ethanol) or 50 μM resveratrol for 48 h. After treatment, apoptosis was assessed using the Annexin V-FITC apoptosis detection kit I (BD Biosciences Pharmingen, San Diego, CA, USA). The cells were washed twice with PBS, suspended in binding buffer and stained with Annexin V-FITC and propidium iodide. Cells undergoing apoptosis were detected by flow cytometry.

Western blot analysis. After treatment, cell lysates were harvested and then subjected to electrophoresis on 7-12% SDS-PAGE gels. Fractionated proteins were electrophoretically transferred onto PVDF membranes. The incubation of the membranes with primary antibodies (anti-caspase-3, anti-caspase-8, anti-PARP, anti-Bcl-2, anti-LC3, anti-beclin-1, anti-mTOR, anti-PI3K, anti-Akt and anti-ERK1/2) was carried out at 4°C. The incubation of the membranes with horseradish peroxidase (HRP)-conjugated secondary antibodies was carried out at room temperature for 1 h, and proteins detected by enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA), as suggested by the manufacturer. After normalization to actin, the control sample was assigned an arbitrary value of 1, and the fold change, in response to resveratrol treatment, was calculated.

Immunofluorescence of LC3. After being incubated with resveratrol (50 μM) for 24 h, the cells were washed with PBS and then fixed with paraformaldehyde (4% w:v). After rinsing in PBS, the cells were blocked with 0.1% Triton X-100 containing 1% bovine serum albumin in PBS for 1 h. This was followed by incubation in goat polyclonal antibody against LC3 for 24 h at 4°C in a humidified chamber. Following 3 washes in PBS, the cells were incubated in donkey anti-goat immunoglobulin G-fluorescein isothiocyanate for 1 h at 4°C. Finally, the cells were rinsed in PBS, coverslipped and examined under a confocal microscope (C1si, Nikon, Tokyo, Japan).

Electron microscopy. The cells were fixed with 2% glutaraldehyde for 2 h, then post-fixed in 1% osmium tetroxide for 1 h. Dehydration was carried out in increasing concentrations of ethanol followed by dehydration in propylene oxide. While being incubated in 70% ethanol, the pellet was stained en bloc with 1% uranyl acetate. Finally, the pellet was embedded in Epon resin. Ultrathin sections were post-stained with uranyl acetate and Reynold's lead citrate routinely. Electron micrographs were acquired using an electron microscope (Hitachi 600, Hitachi, Tokyo, Japan).

Results

Resveratrol suppresses cell viability and induces apoptosis in GH3 cells. We first investigated the effects of resveratrol on GH3 cell viability. The GH3 cells were treated with various doses of resveratrol for 48 h, and cell viability was assessed by MTT assay. The results revealed that resveratrol inhibited GH3 cell viability in a dose-dependent manner. A significant inhibition (62.2%) of cell viability was observed with 12.5 μM, and a maximal inhibition (42.6%) was observed with 100 μM resveratrol (Fig. 1A).

In order to determine the apoptosis induced by resveratrol, the GH3 cells were treated with resveratrol for 48 h. Apoptosis was detected by flow cytometry. Our results revealed that in the control (vehicle-treated) group, apoptosis was observed with a low apoptotic ratio (Fig. 1B); however, in the resveratrol-treated group, resveratrol increased apoptosis in a dose-dependent manner, by approximately 2-fold in the 50 μM resveratrol-treated group compared with the control group (Fig. 1C-E).

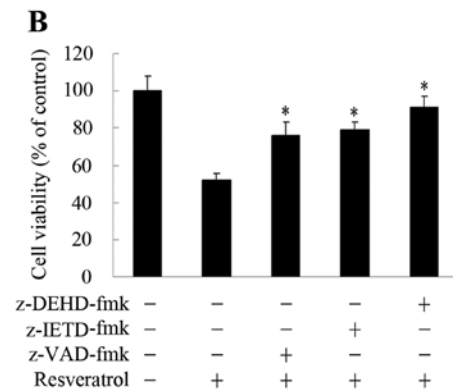
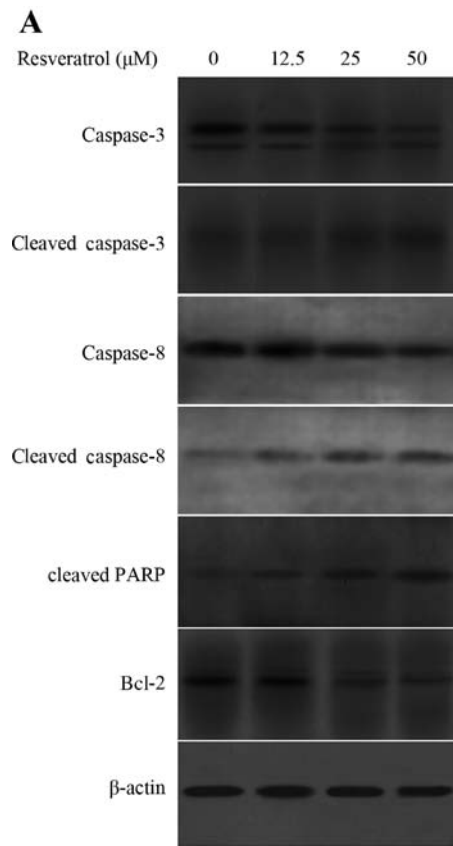


Figure 2. Resveratrol induces the activation of caspases and the reduction of Bcl-2 expression in GH3 cells. (A) GH3 cells were treated with 10, 25 or 50 μM resveratrol for 48 h and then the levels of Bcl-2, PARP, caspase-3 or caspase-8 were detected by western blot analysis. β-actin served as an internal control. (B) GH3 cells were pre-treated with either z-DEVE-fmk (a caspase-3-specific inhibitor), z-IETD-fmk (a caspase-8-specific inhibitor), or z-VAD-fmk (a pan-caspase inhibitor) for 1 h and then treated with 50 μM resveratrol for 48 h. Cell viability was determined by MTT assay. *p<0.05 resveratrol vs. z-DEVE-fmk, z-IETD-fmk and z-LEHD-fmk plus resveratrol (n=3).

Resveratrol-induced apoptosis is caspase-dependent with a decrease in Bcl-2 expression in GH3 cells. To elucidate the mechanisms behind the apoptosis induced by resveratrol, we measured the expression levels of Bcl-2, cleaved PARP, cleaved caspase-3 and caspase-8 following treatment with 50 μM for 48 h. The results revealed that the levels of cleaved PARP and cleaved caspase-3 and caspase-8 were increased in the GH3 cells treated with resveratrol, accompanied by the decreased expression levels of Bcl-2 (Fig. 2A). To further confirm the involvement of caspase activation in resveratrol-induced apop-

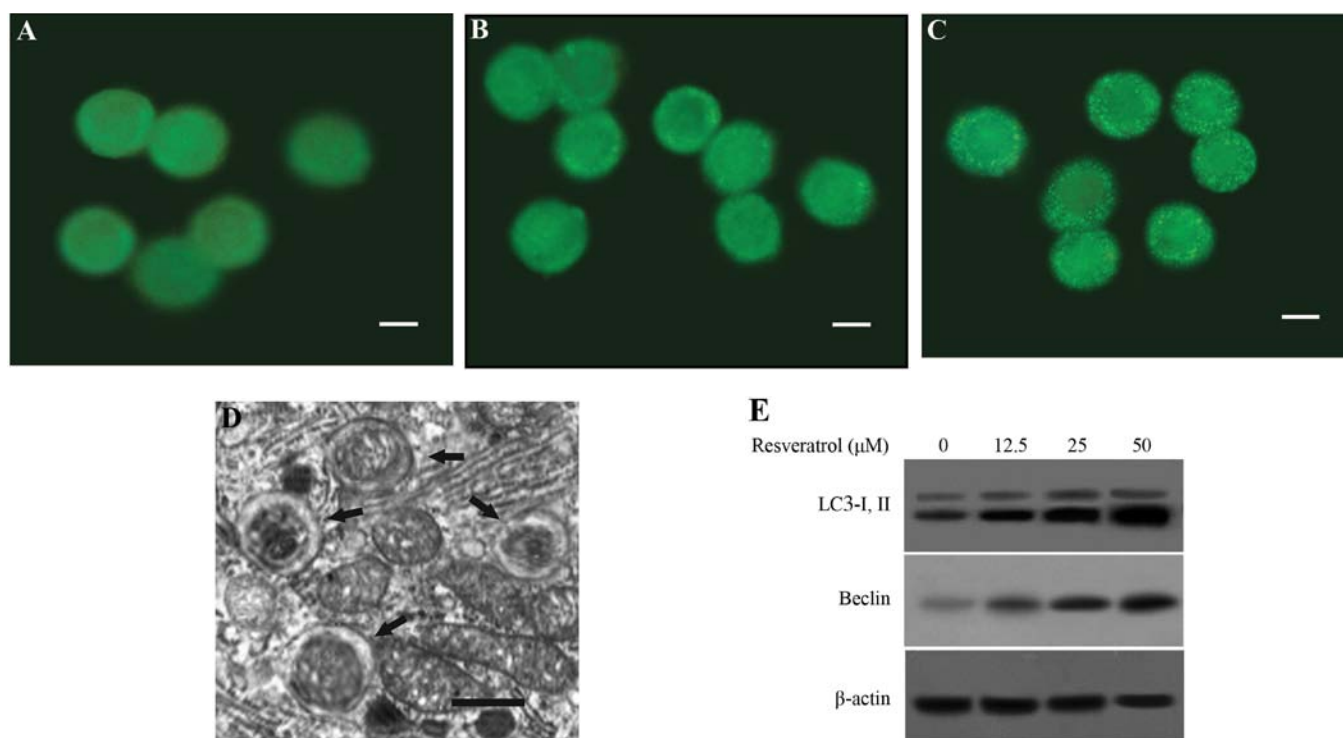


Figure 3. Resveratrol induces autophagy in GH3 cells. GH3 cells were treated with 10, 25 or 50 μ M resveratrol for 48 h. (A–C) Cells were stained with anti-LC3 antibody after treatment. Localization of LC3 was examined under a fluorescence confocal microscope. (A) Negative control: cells were cultured in medium with serum without treatment. (B) Positive control: cells were cultured in serum-free medium without treatment. (C) Treatment group: cells were treated with 50 μ M resveratrol in serum-free medium. Scale bar, 50 μ m. (D) Morphology of autophagy was analyzed under a transmission electron microscope after treatment. Autophagosomes containing cytoplasmic contents are indicated by arrows. Scale bar, 1 μ m. (E) The levels of LC3 and beclin-1 were detected by western blot analysis after treatment. β -actin served as an internal control.

tosis, caspase-specific inhibitors were employed. We found that pre-treatment with z-DEVE-fmk (a caspase-3-specific inhibitor), z-IETD-fmk (a caspase-8-specific inhibitor), or z-VAD-fmk (a pan-caspase inhibitor) effectively attenuated resveratrol-induced cell apoptosis (Fig. 2B). Taken together, these results confirm that resveratrol induces apoptosis through the activation of caspase-3 and caspase-8, and by decreasing the levels of Bcl-2.

Resveratrol induces autophagy in GH3 cells. To determine whether resveratrol induces autophagy in GH3 cells, we observed the localization of LC3, a hallmark of autophagosomes. There are two forms of LC3 proteins: LC3-I and LC3-II. LC3-I is the cytoplasmic form and is converted into LC3-II, which is the autophagosome membrane-bound form. Therefore, the levels of LC3-II correlate with the extent of autophagosome formation (19). LC3 immunoreactivity was examined under a fluorescence microscope. In the control groups, the cells showed only a diffuse distribution of LC3-I immunoreactivity in the presence of serum (Fig. 3A), or with a small amount of scattered LC3-II puncta in the absence of serum (Fig. 3B). However, punctate distribution of LC3-II immunoreactivity was detected at 48 h after resveratrol treatment, representing the increased formation of autophagosomes (Fig. 3C). In addition, autophagosomes, which are double membranous vacuoles containing engulfed cytoplasmic materials, were also observed under an electron microscope (Fig. 3D).

Beclin-1 is required for the formation of autophagic vesicles. It interacts with several cofactors to promote the

formation of beclin-1-Vps34-Vps15 core complexes, thereby inducing autophagy (20). To further confirm the occurrence of autophagy, we determined the levels of beclin-1. We observed that the levels of beclin-1 markedly increased following treatment with resveratrol, indicating autophagy that was induced by resveratrol treatment (Fig. 3E).

Inhibition of autophagy increases caspase activation and enhances apoptosis in resveratrol-treated GH3 cells. To investigate the role of autophagy in resveratrol-induced cell death, 3-MA, an autophagy inhibitor, was employed to detect its effects on resveratrol-induced cell death. The results revealed that treatment with 10 mM 3-MA for 1 h prior to treatment with resveratrol for 48 h led to a decreased level of the resveratrol-induced LC3-II protein, as well as in increased levels of cleaved caspase-8 and cleaved caspase-3 (Fig. 4A). Moreover, pre-treatment with 3-MA reduced cell viability (Fig. 4B) and increased apoptosis (Fig. 4C). In addition, bafilomycin A1 (BafA1), an inhibitor of vacuolar ATPase, which prevents the fusion between lysosomes and autophagosomes at a late stage of autophagy, was also used in the pre-treatment. BafA1 had no significant effects on GH3 cells; however, the resveratrol-induced cytotoxicity was greatly potentiated by BafA1 (Fig. 4B and C). Therefore, autophagy may play an inhibitory role in the apoptotic process in GH3 cells following treatment with resveratrol.

Induction of autophagy by resveratrol is dependent on the regulation of the PI3K/Akt/mTOR and ERK1/2 signaling

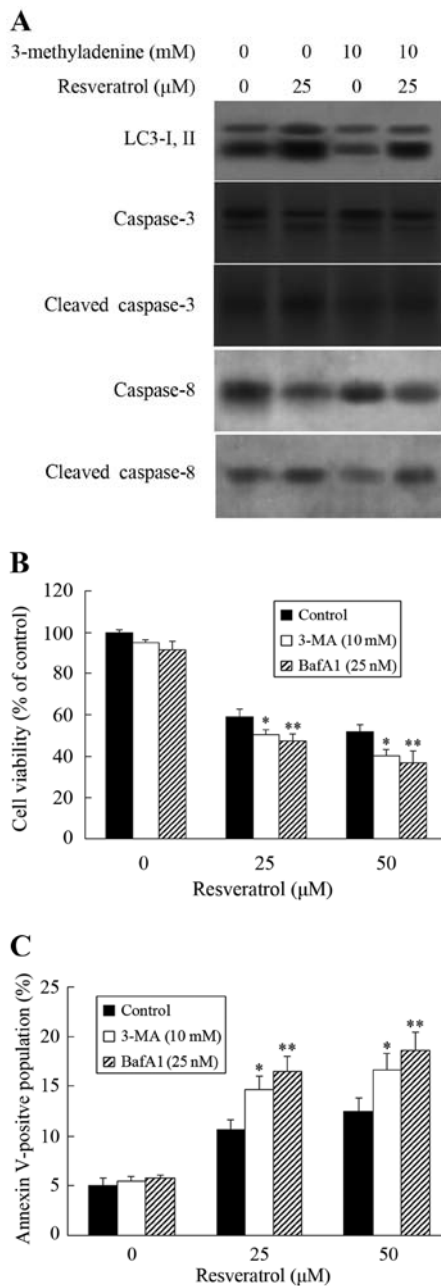


Figure 4. Autophagic inhibitors enhance resveratrol-induced apoptosis. Cells were pre-treated with 10 mM 3-MA or 25 nM bafilomycin A1 (BafA1) for 1 h, and then treated with 25 or 50 µM resveratrol for 48 h. (A) After treatment with 10 mM 3-MA plus resveratrol, the expression of LC3-II, cleaved caspase-3 and cleaved caspase-8 was detected by western blot analysis. β-actin served as an internal control. (B) After treatment, cell viability was determined by MTT assay. *p<0.05, **p<0.05, resveratrol vs. 3-MA or BafA1 plus resveratrol (n=3). (C) After treatment, the cells were harvested and then subjected to quantitative analysis of cell apoptosis by Annexin V and PI double-stained flow cytometry. *p<0.05, **p<0.05, resveratrol vs. 3-MA or BafA1 plus resveratrol (n=3). Control, resveratrol treatment only.

pathways in GH3 cells. Autophagy is regulated by several signaling pathways, through which other cellular processes, such as apoptosis or endoplasmic reticulum stress, interact with each other (21-23). To determine whether the PI3K/Akt/mTOR pathway and the ERK1/2 pathway participate in the autophagy induced by resveratrol, the expression of PI3K, Akt, mTOR and ERK1/2 was examined. The results revealed that, after treatment with resveratrol for 48 h, the expression

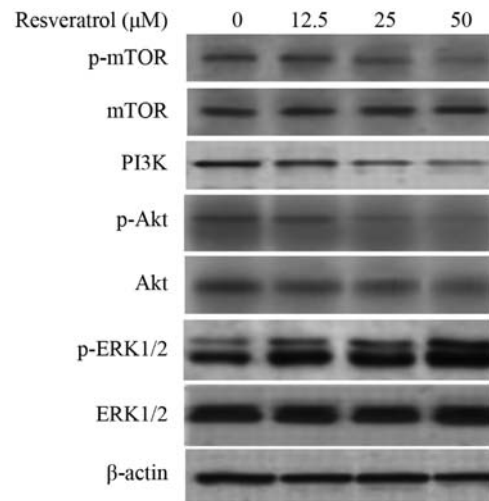


Figure 5. Resveratrol inhibits the activation of the mTOR, PI3K and Akt pathway and activates the ERK1/2 pathway. Cells were treated with 12.5, 25 or 50 µM resveratrol for 48 h. The levels of PI3K, Akt, mTOR and ERK1/2 were detected by western blot analysis after treatment. β-actin served as an internal control.

of PI3K was inhibited and the phosphorylation of Akt and mTOR was decreased. In addition, the phosphorylation levels of ERK were upregulated (Fig. 5). These results demonstrate that resveratrol induces autophagy through the upregulation of ERK1/2 and the downregulation of PI3K/Akt and mTOR phosphorylation.

Discussion

Resveratrol has been identified a potent antitumor agent. A number of studies have revealed the effects of resveratrol on cell growth, inflammation, angiogenesis, tumor invasion and metastasis through multiple intracellular targets (1-3). The present study demonstrates that resveratrol inhibits growth and induces apoptosis in prolactinoma. Previous studies have demonstrated that the resveratrol-induced growth arrest is followed by apoptotic cell death (24-26). Apoptotic cells exhibit increased caspase activity, as detected by the cleavage of the caspase-specific substrate, PARP, that occurs at the onset of apoptosis. Our results revealed a dose-dependent increase in the cleaved PARP fragment, indicating caspase activation (Fig. 3A). Moreover, in the presence of either caspase-3-, caspase-8-specific inhibitor or pan-caspase inhibitor, the resveratrol-induced cytotoxicity was markedly attenuated. These data clearly highlight the role of caspase activation in resveratrol-induced cell death.

The present study demonstrates the induction of autophagy by resveratrol in GH3 cells. It was found that following treatment with resveratrol, LC3-I are converted into LC3-II and the levels of beclin-1 increased. We also observed basal levels of autophagy in the control groups. This possibly results from the serum-free medium, which causes starvation as metabolic stress. Moreover, prolactinoma must balance the extensive production of prolactin with the risk that an excessive load of protein may result in endoplasmic reticulum stress (27), which has been reported to induce autophagy in many types of cancer (21,23). This requires further investigation.

The contribution of autophagy to physiological homeostasis has been previously demonstrated (28). However, an excessive level of autophagy has been suggested to promote cell death due to the overconsumption of critical cellular constituents; this process has been termed ATG-dependent or type II-programmed cell death. Therefore, the outcome of the induction of autophagy can be variable in terms of cell fate and depends not only on the genotype of the cell, but also on the environment (29). It is now well established that autophagy correlates with tumorigenesis, although the exact roles are not yet clear and in some cases, contradictory (11,12). Several anticancer agents have been reported to induce autophagy, and there is a close correlation between resistance to chemotherapeutic agents and autophagy (16-18). Previous studies have suggested that resveratrol induces autophagy, which plays a pro-survival role in multiple cancer cell types (23,30-32). In our study, 3-MA, an autophagic inhibitor, increased the levels of caspase-3 and caspase-8, and enhanced the apoptosis induced by resveratrol. Another specific inhibitor of autophagy, BafA1, increased resveratrol-induced cytotoxicity. These findings suggest that resveratrol-induced autophagy may represent a pro-survival mechanism in GH3 cells. Moreover, in our previous study, we demonstrated that estrogen receptor mediates the effects of resveratrol on GH3 cells (4). Therefore, autophagy may result in antiestrogen resistance in prolactinoma, and agents that inhibit autophagy may be potential candidates for combination treatment with resveratrol.

A number of signaling pathways have been implicated in the regulation of autophagy. Previous studies have suggested that the PI3K/Akt/mTOR pathway and the ERK1/2 pathway regulate autophagy (33,34), and the important roles of these pathways have also been identified in pituitary tumors (35,36). To identify the underlying mechanisms of resveratrol-induced autophagy, the PI3K/Akt/mTOR pathway and ERK1/2 pathway were examined. The results demonstrated that resveratrol inhibited the activation of the PI3K/Akt/mTOR and ERK1/2 signaling pathways (Fig. 5). The results of this study provide evidence that resveratrol-induced autophagy is regulated by the PI3K/Akt/mTOR pathway and ERK1/2 pathway.

Bcl-2 is an antiapoptotic protein that exhibits oncogenic potential through its ability to regulate the apoptotic pathway. Some compounds inhibit the proliferation of prolactinoma by decreasing the levels of Bcl-2 (37,38). In our study, resveratrol attenuated the expression of Bcl-2 followed by the inhibition of proliferation. Beclin-1 was originally identified as a Bcl-2-interacting protein, and binding of the BH3 domain of beclin-1 inhibits PI3K activation, subsequently inhibiting autophagy (20). In cases of nutrient starvation, or when cells are treated with Bcl-2 inhibitors that reduce Bcl-2 protein levels, Bcl-2 and beclin-1 dissociate and autophagy is stimulated (39). In our study, we observed the increase in beclin-1 and the decrease in Bcl-2 levels following treatment with resveratrol. Therefore, through its effects on beclin-1 and Bcl-2, resveratrol influences their binding, and then regulates apoptosis and autophagy. The functional outcome of the interaction between beclin-1 and Bcl-2 is unidirectional, and results in the inhibition of autophagy without having a reciprocal effect on apoptosis (40,41). Autophagy can also directly contribute to the induction of apoptosis. Caspase-8 can be recruited to autophagosomes, which serve as intracellular platforms for caspases

activation (40,41). In the present study, resveratrol induced the activation of caspase-8; however, it remains to be determined whether caspase-8 is involved in the regulation of apoptosis by resveratrol-induced autophagy. Our present results indicate a the crosstalk between autophagy and apoptosis, which possibly mediates the antitumor effects of resveratrol.

In our study, resveratrol inhibited proliferation and induced apoptosis in prolactinoma, and the PI3K/Akt/mTOR pathway and ERK1/2 pathway were implicated in resveratrol induced-autophagy, which plays a pro-survival role. The inhibition of autophagy enhances resveratrol-induced caspase activation and apoptosis. Therefore, resveratrol may act as a novel and potential/promising antitumor agent for prolactinoma.

Acknowledgements

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