

Orexin A induces autophagy in HCT-116 human colon cancer cells through the ERK signaling pathway

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Abstract. Orexins are a class of peptides which have a potent influence on a broad variety of cancer cells. Autophagy is closely associated with tumors; however, its function is not yet completely understood. In this study, we aimed to determine whether orexin A induces autophagy in HCT-116 human colon cancer cells and to elucidate the molecular mechanisms involved. For this purpose, HCT-116 cells were treated with orexin A, and cell viability was then measured by MTT assay, and apoptosis was determined by flow cytometry. The expression levels of autophagy-related proteins were measured by western blot analysis. Quantitative analysis of autophagy following acridine orange (AO) staining was performed using fluorescence microscopy, and cellular morphology was observed under a transmission electron microscope. In addition, the HCT-116 cells were treated with the extracellular signal-regulated kinase (ERK) inhibitor, U0126, or the autophagy inhibitor, chloroquine, in combination with orexin A in order to examine the activation of ERK. We found that orexin A significantly inhibited the viability of the HCT-116 cells. Both autophagy and apoptosis were activated during the orexin A-induced death of HCT-116 cells. When the HCT-116 cells were treated with orexin A for 24 h, an accumulation of punctate microtubule-associated protein-1 light chain 3 (LC3) and an increase in LC3-II protein levels were also detected, indicating the activation of autophagy. Moreover, orexin A upregulated ERK phosphorylation; however, U0126 or chloroquine abrogated ERK phosphorylation and decreased autophagy, compared to treatment with orexin A alone.

Therefore, our findings demonstrated that orexin A induced autophagy through the ERK pathway in HCT-116 human colon cancer cells. The inhibition of autophagy may thus prove to be an effective strategy for enhancing the antitumor potential of orexin A as a treatment for colon cancer.

Introduction

Autophagy is a conserved eukaryotic cellular degradative process that is indispensable in cell homeostasis (1). Autophagy ameliorates cellular damage during stress conditions by eliminating damaged cellular machinery, aged organelles and unwanted macromolecules, and recycling them to produce essential nutrients and energy for reuse (2,3). On the contrary, failure in any part of the autophagic machinery leads to the development of a variety of diseases, such as cancer (4-6), neurodegenerative diseases (7), heart diseases (8) and autoimmune diseases (9). Consequently, the identification and/or development of autophagy-modulating compounds has become a new research goal for the treatment of these diseases.

The orexin peptides (orexin A and B) and their receptors (orexin receptor type 1 and 2) are involved in the regulation of numerous physiological processes, such as sleep/wakefulness (10), breathing (11), the reward system (12) and drug addiction (12,13). Previous research has reported that orexin A markedly induces apoptosis, resulting in a reduction in cell growth in various cancer cell lines. In a previous study, orexin A promoted the apoptosis of HT29-D4 colon cancer cells, but not that of normal colonic epithelial cells (14). Upon treatment with orexins, Chinese hamster ovary (CHO) cells transfected with orexin receptor type 1 (OX1R) cDNA underwent growth suppression and apoptosis, as did SK-N-MC neuroblastoma cells transfected with endogenous OX1R (14). In addition, it has been observed that OX1R receptors induced cell death via the p38 mitogen-activated protein kinase (MAPK), but independently of p53 and caspase activation in CHO cells (15). In another study, rat C6 glioma cells expressing both OX1R and orexin receptor type 2 (OX2R) were treated with orexin A, which suppressed the growth of the cells through a caspase-dependent mechanism (16). However, programmed cell death is generally divided into apoptosis, autophagy and necrosis (17). Studies have mainly focused on the promotion of cell apoptosis by orexin A (14,16); however, to date and to the best of our knowledge, no study has addressed the theory

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that orexin A can induce autophagy in cancer cells. Thus, in the present study, we investigated the effects of orexin A on autophagy in HCT-116 human colon cancer cells.

The extracellular signal-regulated kinase (ERK) controls various cellular processes, including autophagy. It has been shown that growth factor increases the interaction of the ERK cascade components with autophagy-related (ATG) proteins in both the cytosol and nucleus. ERK and its upstream kinase, MAPK/ERK kinase (MEK), localize to the extra-luminal face of autophagosomes, and lipidation of the autophagic protein, microtubule-associated protein-1 light chain 3 (LC3) upregulates ERK phosphorylation (18). The enhanced activity of ERK1/2 has been implicated in controlling the induction of autophagy in mammalian cancer cells (19). Based on these previous findings, in this study, we aimed to determine whether the MEK/ERK pathway plays a role in orexin A-induced autophagy.

In the present study, we hypothesized that a membrane-associated pathway is involved in orexin A-induced autophagy, and to prove this hypothesis, we used HCT-116 human colon cancer cells as a model. We observed that orexin A induced autophagy in the HCT-116 cells. Moreover, it was demonstrated that in the presence of an ERK inhibitor and an autophagy inhibitor, the orexin A-induced autophagy was inhibited, which indicated that orexin A induced autophagy through the ERK pathway. The findings of our study demonstrate a different mechanism responsible for the activation of the autophagic pathway induced by orexin A.

Materials and methods

Reagents. Orexin A was purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). Acridine orange (AO) and the autophagy inhibitor, chloroquine, were obtained from Sigma-Aldrich. The ERK inhibitor, U0126, was purchased from Beyotime Biotechnology (Jiangsu, China). Anti-actin (#sc-1616) and anti-ERK (#sc-292838) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-Beclin-1 antibody (#B6061) was purchased from Sigma-Aldrich. Anti-LC3 (#L7543) and anti-p-ERK (#4370s) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. HCT-116 human colon cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in RPMI-1640 medium supplemented with FBS (10% w/v), L-glutamine (2 mM), penicillin (50 µg/ml) and streptomycin (100 µg/ml) (Beyotime Biotechnology). The cells were incubated in a humidified atmosphere containing 5% carbon dioxide (CO₂) at 37°C. The cells were subcultured every 2-3 days to maintain the logarithmic phase of their growth.

Cell viability. The HCT-116 cells were seeded (2x10³ cells/well) on a 96-well plate and cultured for 24 h. Following incubation in serum-free RPMI-1640 medium supplemented with orexin A (0, 10⁻⁹, 10⁻⁸ and 10⁻⁷ M) for 24 h, thiazolyl blue tetrazolium blue (MTT) solution (0.5 mg/ml) was added (Sigma-Aldrich). After 3 h, the culture medium was removed and formazan crystals that had formed were dissolved using

100 µl dimethyl sulfoxide (Merck KGaA, Darmstadt, Germany). Optical density was measured using a plate reader (SpectraMax Plus 384 Microplate Reader; Molecular Devices, Ismaning, Germany) at 570 and 650 nm (reference wave/length).

Annexin V/propidium iodide (PI) assays for the measurement of apoptosis. The number of apoptotic cells was quantified using the Annexin V/PI Apoptosis Detection kit and the cells were evaluated to determine apoptosis using a BD Accuri™ C6 Flow Cytometer according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). The cells were treated with various concentrations of orexin A in serum-free medium for 48 h. The cells (1x10⁵) were then washed twice with phosphate-buffered saline (PBS) and stained with Annexin V-fluorescein isothiocyanate (FITC; 5 µl) and PI (10 µl) in 500 µl binding buffer for 15 min at room temperature in the dark. The rate of apoptosis was determined by counting the number of cells stained by FITC-labeled Annexin V by fluorescence-activated cell sorting (FACS) analysis. The early apoptotic cells were identified as PI-negative and FITC-Annexin V-positive; cells that were in late apoptosis or already dead were PI- as well as FITC-Annexin V-positive.

Transmission electron microscopy. The cells were treated and collected by trypsinization, fixed with 2.5% phosphate-buffered glutaraldehyde, and post-fixed in 1% phosphate-buffered osmium tetroxide. The cells were embedded, sectioned, double stained with uranyl acetate and lead citrate, and analyzed using a JEM-1200EX transmission electron microscope (TEM; JEOL, Tokyo, Japan).

AO staining. AO (0.1 mg/ml) was added to the cells following treatment with various concentrations of orexin A (0, 10⁻⁹, 10⁻⁸ and 10⁻⁷ M) for 24 h, or to the untreated controls cells for a period of 20 min in the dark at 37°C. The cells were washed twice with PBS. The cells were then examined under a fluorescence microscope (Olympus Optical Co., Hamburg, Germany).

Flow cytometric quantification of acidic vesicular organelles (AVOs). For the quantification of AVOs, flow cytometry was used following the staining of the cells with AO. AO is a weak base that accumulates in the acidic spaces and imparts bright red fluorescence [punctate staining (dots)] in the cytoplasm, which is detected under a fluorescence microscope. The intensity of the red fluorescence is proportional to the degree of acidity. Thus, the formation of AVOs can be quantified. Briefly, the HCT-116 cells were harvested following treatment with various concentrations of orexin A (0, 10⁻⁹, 10⁻⁸ and 10⁻⁷ M) for 24 h. The cell pellet was collected in an Eppendorf tube, and the cells were resuspended in PBS (1 ml). The staining of the cells was performed with AO (0.1 mg/ml) for 20 min in the dark at 37°C. The cells were centrifuged at 1,000 rpm for 5 min; the cell pellet was then rinsed twice with PBS, resuspended in PBS (500 µl), and analyzed by flow cytometry using the PI staining assay. Cell death was determined by PI staining.

Protein preparations and western blot analysis. The HCT-116 cells were washed with cold PBS and harvested

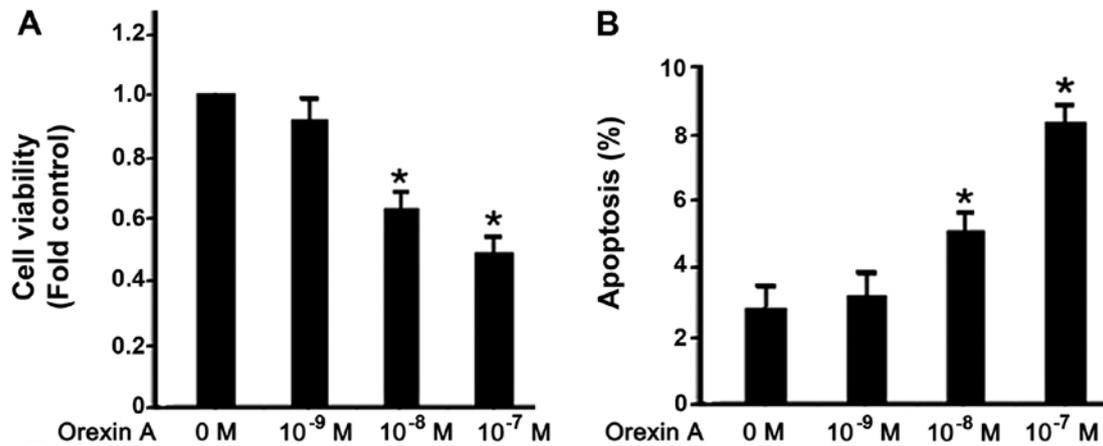


Figure 1. Effects of orexin A on the viability and apoptosis of HCT-116 cells. HCT-116 cells were treated with orexin A (0, 10⁻⁹, 10⁻⁸ and 10⁻⁷ M) for 24 h. (A) Cell viability was determined by MTT assay. (B) Cell apoptosis was determined by flow cytometry followed by Annexin V staining. Data are presented as the means ± SEM based on triplicate determinations from a representative experiment. Asterisks indicate significant differences as compared to the untreated control (*P<0.05).

in radioimmunoprecipitation assay buffer (Beyotime Biotechnology) containing protease inhibitors, namely phenylmethylsulfonyl fluoride (Beijing, Jiangsu, China) and phosphatase inhibitors (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The cell lysates were incubated on ice for 30 min, and were then collected and centrifuged at 12,000 × g for 10 min at 4°C. The supernatants were collected and mixed with 5X loading buffer, and were denatured by boiling for 10 min. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes at 60 V for 2.5 h in a transfer buffer containing Tris (20 mM) (bioWORLD, Dublin, OH, USA), 150 mM glycine (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and methanol (20%) (Liaoning Xinxing Chemical Group Co., Ltd., Liaoning, China). The membranes were incubated in non-fat dry milk for 120 min at room temperature, and were washed thrice with Tris-buffered saline with Tween 20 (TBST) for 30 min. The membranes were incubated with primary antibodies in TBST overnight at 4°C. The membranes were then washed and incubated with horseradish peroxidase-conjugated anti-species secondary antibody (A0208; Beyotime Biotechnology) for 1.5 h at room temperature, and were washed thrice with TBST for 30 min. Proteins were visualized using the BeyoECL plus kit (Beyotime Biotechnology).

Statistical analysis. Data are expressed as the means ± standard error of the mean (SEM), and the differences between the means were analyzed by one-way analysis of variance (ANOVA). A P-value <0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 15.0 software package (SPSS, Inc., Chicago, IL, USA).

Results

Orexin A inhibits cell viability and induces apoptosis. The HCT-116 cells were treated with various concentrations of orexin A (0, 10⁻⁷, 10⁻⁸ and 10⁻⁹ M). The cells were serum-starved for 24 h prior to exposure to the test compounds in

order to avoid interaction with growth factors and other mediators present in the serum. The results from the MTT assay revealed that orexin A inhibited cell growth in a dose-dependent manner. Treatment with orexin A at the concentrations of 10⁻⁸ and 10⁻⁷ M resulted in a significant decrease in the viability of the HCT-116 cells as compared to the untreated control (P<0.05; Fig. 1A). The results of flow cytometry indicated a significant increase in apoptosis in the cells treated with orexin A for 24 h. Thus, the present study demonstrated that treatment with orexin A (10⁻⁷ M) increased the cell apoptotic rate by 1.5-fold, as compared to the untreated control (P<0.05; Fig. 1B). These findings indicate that orexin A inhibits HCT-116 cell viability by inducing apoptosis.

Formation of autophagic vacuoles and AVOs in HCT-116 human colon cancer cells. To further elucidate the anticancer effects of orexin A, we examined other cellular responses associated with cell death following treatment of the cells with orexin A. In contrast to the untreated control group, numerous microscopic vacuoles were observed in the HCT-116 cells treated with orexin A (10⁻⁷ M) (Fig. 2). Since the formation of double-membrane autophagic vacuoles is a characteristic of autophagy, we focused on examining the ultrastructural details of the vacuoles using a TEM. As shown in Fig. 2A, various membrane-associated vacuoles were observed in the cytoplasm of the HCT-116 cells treated with orexin A. To further characterize the membrane-associated vacuoles, AO staining was used to analyze the formation of AVOs, which is a characteristic of autophagy. Orexin A induced the formation of orange AVOs in a dose-dependent manner in the HCT-116 cells, whereas the cells in the untreated control group primarily exhibited green fluorescence, indicating a lack of AVOs (Fig. 3A-D). The orexin A-induced formation of AVOs was quantified by flow cytometry after staining the cells with AO. The results from the flow cytometry demonstrated a dose-dependent increase in the percentage of AVOs from 0.8 to 21% (Fig. 3E-H).

Orexin A induces LC3-II accumulation. We demonstrated that orexin A induced autophagy in HCT-116 cells, an obser-

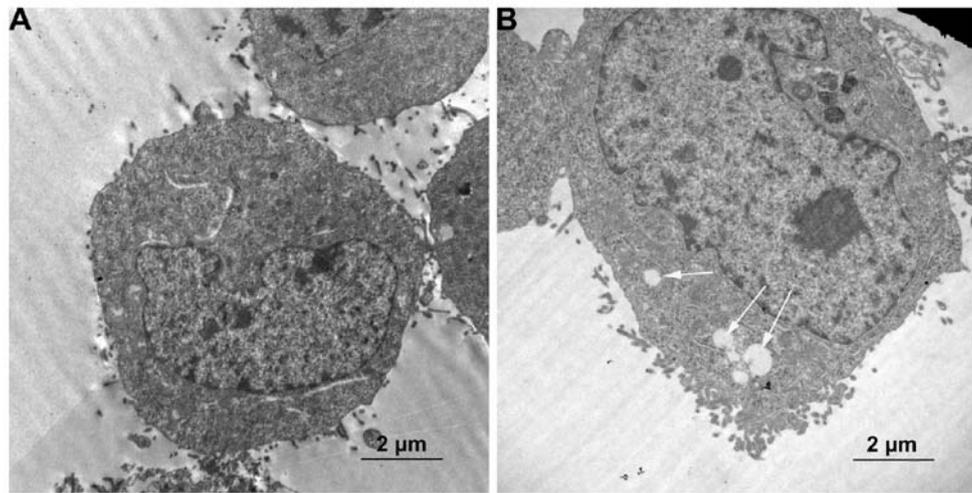


Figure 2. Orexin A induces the formation of autophagic vacuoles in HCT-116 cells. (A) Untreated HCT-116 cells exhibited normal morphology of the cytoplasm, cell organelles and nuclei. (B) Transmission electron microscopy images indicating autophagic vacuoles in the HCT-116 cells treated with 10^{-7} M orexin A for 24 h. Arrows indicate autophagic vacuoles.

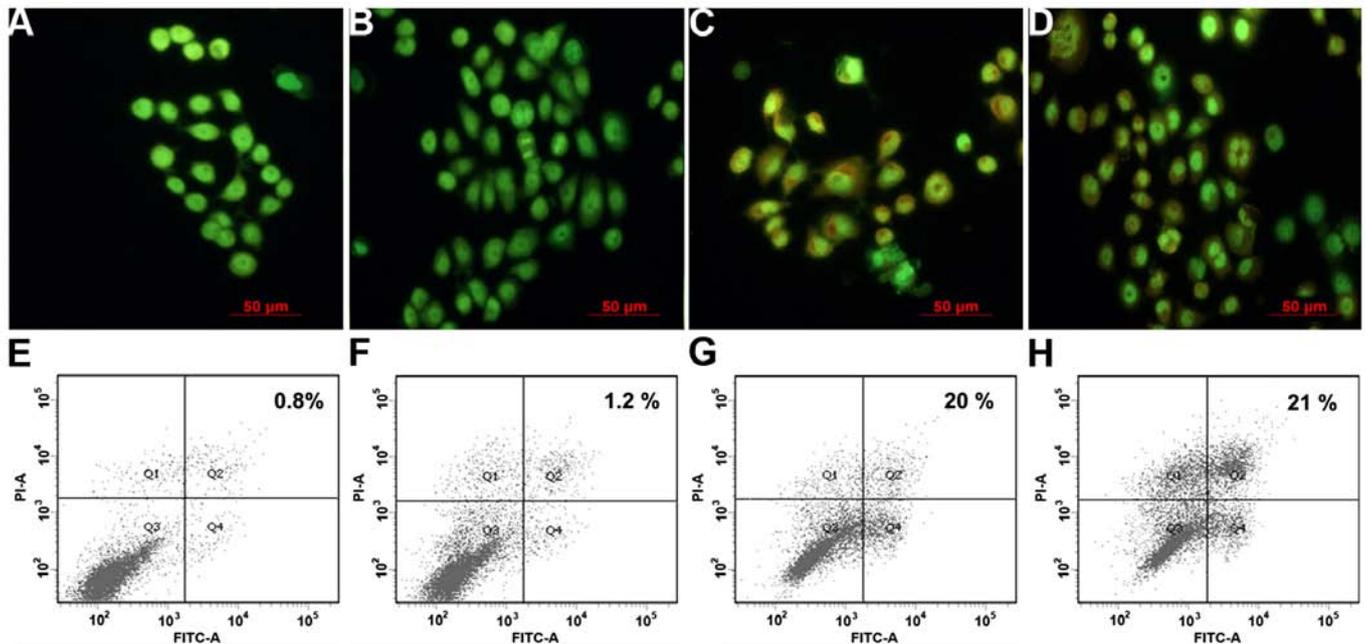


Figure 3. Orexin A induces the formation of autophagic vacuoles and acidic vesicular organelles (AVOs) in HCT-116 cells. (A-D) Representative images of acridine orange (AO) staining of HCT-116 cells following treatment with various concentrations [(A) 0, (B) 10^{-9} , (C) 10^{-8} or (D) 10^{-7} M] of orexin A for 24 h. For AO staining, red color intensity indicates AVOs (representing autophagolysosomes). HCT-116 cells were treated with orexin A and were stained with AO. (E-H) Cell autophagy was analyzed by the quantification of AVOs with AO staining by flow cytometry. HCT-116 cells were treated with various concentrations [(E) 0, (F) 10^{-9} , (G) 10^{-8} or (H) 10^{-7} M] of orexin A for 24 h. Representative data from one of three independent experiments are shown.

vation which was based on the ultrastructural changes in the cells as observed by a TEM and the visualization of acridine orange-labeled autophagic vacuoles under a fluorescence microscope. Thus, we further examined the expression levels of LC3, which exists in cells in two forms: LC3-I and LC3-II. LC3-I resides in the cytoplasm and conjugates with phosphatidylethanolamine to form LC3-II, which is closely associated with autophagosome membranes and serves as a reliable marker for autophagy. Western blot analysis of the proteins obtained from the orexin A-treated cells revealed the pres-

ence of two bands. Weak bands corresponding to LC3-I and LC3-II were observed in the untreated cells. When the cells were treated with orexin A (10^{-7} M), the expression levels of LC3-II were significantly increased in a time-dependent manner (Fig. 4A). The exposure of the HCT-116 cells to orexin A for 24 h also resulted in a concentration-dependent increase in the LC3-II protein levels (Fig. 4B).

Orexin A upregulates the expression of Beclin-1 in the HCT-116 cells. Based on the above-mentioned results, we speculated that

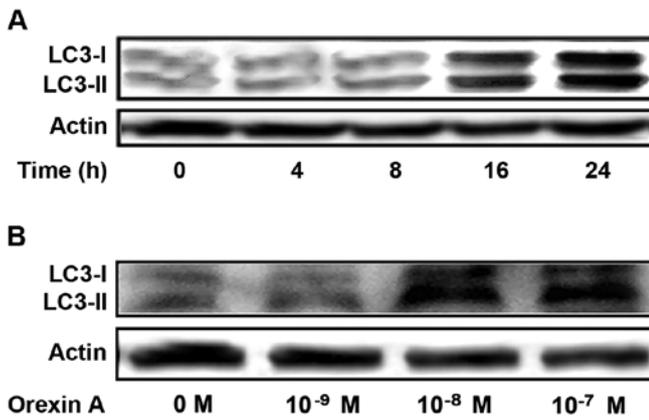


Figure 4. Orexin A induces LC3-II accumulation in HCT-116 cells. (A) Western blot analysis of LC3 expression in HCT-116 cells treated with 10^{-7} M orexin A for the indicated periods of time. (B) HCT-116 cells were treated with various concentrations of orexin A for 24 h. The protein expression of LC3 was measured by western blot analysis. Actin was used as a loading control. All data are representative of three independent experiments.

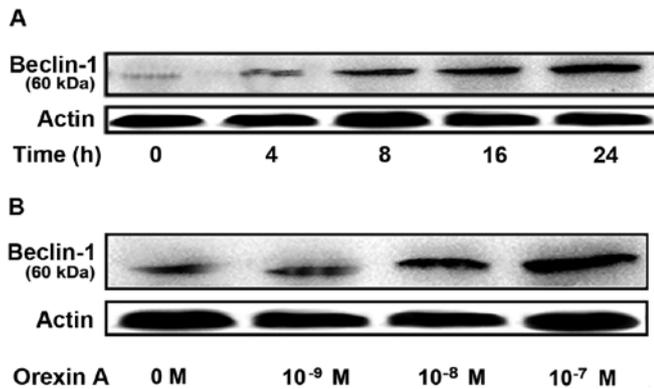


Figure 5. Orexin A activates the autophagy-related gene Beclin-1 in HCT-116 cells. HCT-116 cells were treated with various concentrations of orexin A for the indicated periods of time. The protein activation of Beclin-1 (~60 kDa) was normalized against the total protein activation. Actin was used as an internal control. Data are presented as the means \pm SEM based on triplicate determinations from a representative experiment.

orexin A would induce HCT-116 cell death through the apoptosis and autophagic pathways simultaneously. To gain better insight into the molecular mechanisms underlying the autophagy and apoptosis induced by orexin A, the expression levels of Beclin-1, which plays a key role in autophagy in HCT-116 cells treated with orexin A, were measured by western blot analysis. As shown in Fig. 5, orexin A upregulated the expression of Beclin-1 in a dose- and time-dependent manner. These findings indicate that the upregulation of Beclin-1 may contribute to the induction of autophagy caused by orexin A.

Orexin A induces autophagy in HCT-116 cells through the ERK signaling pathway. In order to confirm the involvement of the ERK signaling pathway in orexin A-induced autophagy in HCT-116 cells, the protein expression levels of LC3 and Beclin-1 were measured by western blot analysis. The results revealed that treatment with 10^{-7} M orexin A significantly increased the expression levels of LC3-II and Beclin-1 (Fig. 6).

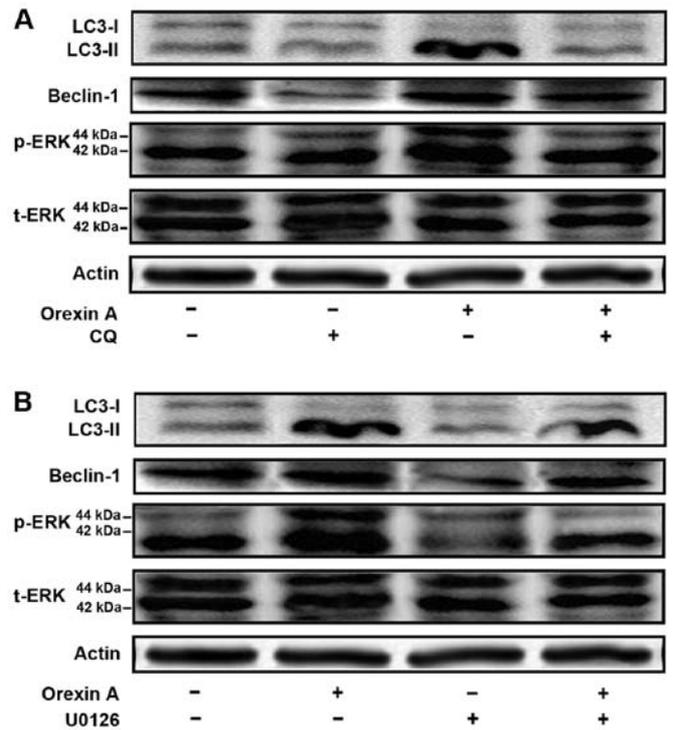


Figure 6. Orexin A induces autophagy in HCT-116 cells through the activation of the ERK signaling pathway. (A) Representative blots of p-ERK, ERK1/2, Beclin-1 and LC3 protein expression following treatment of the cells with either 10^{-7} M orexin A or 20 μ mol/l chloroquine (CQ) for 24 h. Cell lysates were analyzed by western blot analysis using anti-p ERK, ERK1/2, Beclin-1 and LC3 antibodies. (B) Cells were exposed to orexin A at concentrations of 0 and 10^{-7} M for 24 h in the presence or absence of the ERK-specific inhibitor, U0126 (10^{-5} M). Cell lysates were analyzed by western blot analysis using anti-p-ERK, ERK1/2, Beclin-1 and LC3 antibodies. Data shown represent the means \pm standard error of the mean (SEM) of three independent experiments.

However, this effect was blocked by treatment with 10^{-5} M of the ERK antagonist, U0126, or with 20 μ mol/l chloroquine (autophagy inhibitor) (Fig. 6). Moreover, the expression levels of LC3-II and Beclin-1 were not significantly altered when the cells were treated with the ERK antagonist, U0126, or chloroquine without orexin A treatment (Fig. 6). These data suggest that ERK participates in the autophagy of HCT-116 cells induced by orexin A.

It is well known that the ERK signaling pathway is involved in cell autophagy and apoptotic signaling; therefore, we also wished to investigate whether the treatment of HCT-116 cells with orexin A induces the activation of ERK. Our results revealed a 1.5-fold increase in p-ERK protein expression in the HCT-116 cells treated with 10^{-7} M orexin A compared to the untreated control cells (Fig. 6). However, the total ERK (t-ERK) levels remained unaffected by the aforementioned treatment. Moreover, treatment with 10^{-5} M of the ERK antagonist, U0126, or with 20 μ mol/l chloroquine (autophagy inhibitor) abolished the relative increase in ERK activation in response to orexin A independently (Fig. 6).

Discussion

It has previously been demonstrated that orexin A is involved in a wide range of biological activities (10-13). In particular,

orexin A has been reported to exert anti-proliferative and apoptotic effects on cancer cells (20,21). Programmed cell death is generally divided into apoptosis, autophagy and necrosis. Previous studies have focused on the promotion of cell apoptosis by orexin A (14,16), and a few have reported on the promotion of autophagy by orexin A. To the best of our knowledge, there is no study available to date addressing the theory that orexin A induces autophagy in cancer cells. In this study, we investigated the effects of orexin A on autophagy in HCT-116 human colon cancer cells.

Autophagy can be induced under various circumstances, including aging, nutrient deprivation, as well as chemical therapy and radiation cancer therapy (3,22). Autophagy is a process of catabolism through which macromolecules and organelles are recycled using lysosomal degradation machinery (3,23). Autophagy begins with the formation of double-membraned vesicles known as autophagosomes, which undergo acidification after maturation and subsequently fuse with lysosomes to form autophagolysosomes (24). Autophagy likely plays different roles in the occurrence and progression of cancer, while it also potentially promotes or inhibits cell proliferation at different stages of tumor growth (25). For example, autophagy plays a protective role in tumor cells via the degradation of organelles under conditions of nutritional deficiency. Conversely, autophagy can also inhibit tumor growth via Beclin-1, UV radiation resistance associated gene (UVRAG), Bif and ATG proteins (26). In the present study, we observed that orexin A inhibited the proliferation and induced the apoptosis of HCT-116 cells. Moreover, treatment of the cells with orexin A induced changes to the cells which are characteristic of autophagy, such as the formation of cytoplasmic autophagic vacuoles and autophagosomes. It was evident that AVOs formed following the exposure of the cells to orexin A, which was shown by AO staining. The formation of autophagosomes was further confirmed by transmission electron microscopy. Additionally, the conversion of LC3-I to LC3-II induced by orexin A was also shown by western blot analysis, and we also noted that LC3 expression increased in a dose- and time-dependent manner following treatment with orexin A. These specific changes of LC3 have been characterized as an autophagosomal marker in mammalian autophagy. Our results demonstrated that orexin A induced autophagy in HCT-116 human colon cancer cells.

We further examined the protein expression levels of Beclin-1 in the HCT-116 cells. Beclin-1 is a part of the class III phosphatidylinositol 3-kinase (PI3K) complex, which is essential for the initiation of the early stages of autophagy. Beclin-1 expression is commonly decreased or absent in various cancer cells, such as human breast carcinoma, ovarian cancer, brain tumors, cervical cell carcinoma and gastric cancer (27-31). These findings demonstrate that Beclin-1 is a critical component of mammalian autophagy. In the present study, treatment of the HCT-116 cells with orexin A increased the expression of Beclin-1 in a dose-dependent manner, indicating that Beclin-1 is involved in orexin A-induced autophagy and plays an important role in orexin A-mediated anticancer activities.

ERK is a versatile protein kinase that regulates a number of cellular functions. It is considered that the magnitude and duration of ERK1/2 activity determine its cellular function (32). It has also been reported that the activation of the

ERK1/2 pathway increases the expression of autophagic genes, which can lead to an increase in the autophagic flux (33,34). An increase in p-ERK 1/2 and ATG protein LC3-II expression has also been observed in human glioma cells treated with interferon β (IFN- β). The autophagy induced by IFN- β was suppressed when p-ERK1/2 was inhibited by treatment with U0126 (35). U0126 is a chemically synthesized organic compound, which inhibits the catalytic activity of the activating enzyme through non-competitive binding to MEK, thereby decreasing the phosphorylation of ERK1/2 (36,37). Certain antitumor drugs induce autophagy and activate the ERK1/2 signaling pathways in human lung cancer cells (38). In the present study, we found that orexin A significantly enhanced the phosphorylation of ERK, and we also noted that the p-ERK inhibitor, U0126, reduced the orexin A-induced increase in LC3-II expression. To further investigate the effects of orexin A on cell autophagy through the ERK pathway, we used the autophagy inhibitor, chloroquine as a reference. Chloroquine blocks the process of autophagy by blocking autophagic bodies fused with lysosomes, and thus cells cannot be provided with raw materials or energy (39). Our results revealed that the ERK inhibitor and autophagy inhibitor significantly inhibited the promoting effects of orexin A on autophagy, indicating that orexin A induced autophagy through the ERK pathway.

In conclusion, in the present study, we explored the antitumor effects and the underlying mechanisms of action of orexin A on colon cancer cells. Our data lay the experimental foundation for the better development and clinical application of orexin A.

Acknowledgements

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