Atropine can induce autophagy independent of the M3 muscarinic acetylcholine receptor

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Abstract
Background: No other effects of atropine other than as an antagonist of muscarinic acetylcholine receptor (mAChR) have been found. Methods: In this study, human kidney epithelial cells were treated with different physiological regulators. Results: Subsequently, it was found that atropine could significantly induce autophagy as demonstrated by the appearance of autophagosome-like double- or single-membrane vesicles in the cytoplasm of host cells and the number of GFP-LC3 dots. In addition, increased conversion of the autophagy marker protein LC3-I and LC3-II and increased p62/SQSTM1 indicated incomplete autophagy. In addition, atropine induced autophagosome levels in a dose-dependent manner within a certain concentration range in human kidney epithelial cells. In atropine-treated mouse skeletal muscle cells containing nicotinic acetylcholine receptors and rat cardiac muscle cells containing mAChR, atropine induced autophagy in mouse skeletal muscle cells but not in rat cardiac muscle cells. Furthermore, atropine did not induce autophagy in tissue cells containing mAChR in vivo but did in tissue cells not containing mAChR. Conclusion: This study expands the application and understanding of atropine’s action mechanism in the field of medicine.

Keywords: autophagy; atropine; acetylcholine receptor; cells
Highlights
Atropine can induce autophagy as an agonist of muscarinic acetylcholine receptor by binding to other receptors. This study expands the application and understanding of atropine’s action mechanism in the field of medicine.

Medical history of objective
Atropine is a compound found naturally in plants such as belladonna, mandala, and hypane, and has been used by civilizations around the world to treat pain and other symptoms. Mandala was first recorded in Li Shi-Zhen’s Compendium of Materia Medica (1552–1578 C.E.), which has anesthetic effects.

Background
Autophagy is a catabolic process that captures and degrades damaged proteins and organelles in lysosomes. This mechanism may be caused by cell physiological stressors, including hypoxia, hunger, endoplasmic reticulum stress, metabolic stress, lack of nutrition, or extremely low pH [1, 2]. Ghrelin, a novel gut hormone, has been shown to exert its cardioprotective effects on cardiac hypertrophy by promoting autophagy, possibly via CaMKK/AMPK signaling pathway [3]. In addition, ghrelin can inhibit NLRP3 inflammasome activation by upregulating autophagy to improve Alzheimer’s disease [4]. Fu et al. reported that glucocorticoids induce autophagy enhancement of primary osteoclasts through the PI3K/Akt/mTOR signaling pathway [5]. Hong et al. found that the autophagy-lysosome pathway is one specific molecular mechanism in regulating Aβ-induced NLRP3-caspase-1 inflammasome activation in astrocytes. In particular, they uncovered the potential neuroprotection of progesterone in regulating upstream signaling leading to the sequence events of neuroinflammation and that neuroprotective mechanism of progesterone in regulating NLRP3-caspase-1 inflammasome can be a potential therapeutic target for ameliorating the pathological procession of Alzheimer’s disease [6].

Atropine is an anti-muscarinic drug, and its enhanced parasympathetic nerve suppression promotes sympathetic nerve stimulation by competitively inhibiting the vagus nerve and postganglionic acetylcholine receptors, leading to increased cardiac output and other related anti-muscarinic side effects [7, 8]. Atropine can be used for parenteral emergency during anesthesia, including cardiac arrhythmia, to prevent vagal reflexes, reduce secretions, acute bronchospasm, and anticholinesterase overdose or poisoning [9]. According to different studies conducted on animals and humans, this alkaloid has been found to be widely distributed in tissues [10], and has a binding affinity constant in the range of 0.4–0.7 nM for all five subtypes of muscarinic acetylcholine receptors (M1 to M5) [11, 12]. In regard to treatment, atropine has a wide range of indications [13]. Atropine is used as a pre-medication for anesthesia and surgery, as an anti-salivary agent to suppress salivation and excessive secretions, and as an anti-vagal agent to prevent cholinergic action during surgery [14]. In addition, atropine can reverse muscle relaxation [15]. Atropine is also an accidental or intentional antidote to poisoning by anti-cholinesterase agents, such as organophosphorus insecticides, carbamates, or mushrooms containing muscarinic acid. In this case, atropine competes with acetylcholine as a receptor in muscarinic acid, thereby preventing their activation and reversing bronchial leakage and bronchoconstriction. Because atropine does not bind to nicotinic acetylcholine receptors (nAChR), it is not effective in reversing nicotinic symptoms such as muscle weakness, convulsions, and paralysis [16].

Atropine has been used in medicine widely, and apart from its role as a muscarinic acetylcholine receptor (mAChR) antagonist, no other effects of this molecule have been found. It has been reported that atropine inhibits epilepsy-induced autophagy by antagonizing mAChR, while we have previously found that atropine may induce autophagy in cells that do not express mAChR [17, 18]. In this study, we try to investigate the effect of atropine on autophagy in the presence or absence of mAChR and provide a theoretical basis for the development and application of atropine.

Materials and methods
Cells
Cells from the human kidney epithelial (293T) cell line, mouse skeletal muscle (C2C12) cell line, and rat cardiac muscle (H9c2) cell line were preserved in our laboratory, were cultured at 37 °C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 U/mL streptomycin.

RNA interference of mAChR
In order to explore whether the effect of atropine on autophagy depends on the antagonism of atropine, the expression of mAChR in H9c2 cells was silenced using small interfering RNA (siRNA) targeting the rat mAChR gene (GenePharma Co., Ltd., Shanghai, China). The sequence of si-mAChR-1 was AGACAGUUAACACAUCC (sense) and UAGUGAUGUGUAACUGUGUC (antisense); the sequence of si-mAChR-2 was AGCUCAGAGACAGUUAACACUC (sense) and UUGUUAACGUGCUAGAGUGA (antisense); the sequence of si-mAChR-3 was AGCUCAGACAGUUAACACACU (sense) and UUAACUGUCUUGAGGUACUG (antisense). Six-well plates were transfected with siRNAs and negative control RNA using transfection reagent for 24 h and then treated with atropine. Cell samples were collected at 48 h to test the silencing effect and autophagy levels.

Drug treatment
Epinephrine, insulin, atropine, neostigmine, furomeside, and posterior pituitary hormone (Hofeng Pharmaceutical Co., Ltd., Shanghai, China), all at concentrations of 0.5 mg/mL, were used to treat 293T cells in six-well culture plates for 24 h. Cells were then collected and lysed to obtain protein samples and analyze the conversion of LC3 (microtubule-associated protein light chain) 1 to LC3II. Atropine (Beyotime, Shanghai, China), at concentrations of 0.0005 mg/mL, 0.005 mg/mL, 0.01 mg/mL, and 0.5 mg/mL, was used to treat 293T cells in six-well culture plates for 24 h to analyze the conversion of LC3I to LC3II. Furthermore, 200 μM of rapamycin was used to treat C2C12 cells and H9c2 cells in six-well culture plates for 2–4 h as a control.

In vivo experiments
Twenty SPF 7-week-old 20–22 g Kunming male mice (Changsheng Biological Co., Ltd., Bexin, China) (license number: SCXK (Liao) 2020-0001) were divided into an atropine group and a phosphate-buffered saline (PBS) group, raised under the same conditions of food and water supply in the same temperature environment. Sterile 1× PBS and atropine at a concentration of 0.5 mg/mL were injected intraperitoneally in their respective groups at a dose of 10 μL per 1 g body weight every day for 14 days. Mice were euthanized and their hippocampal tissue and smooth muscle tissue were lysed to obtain protein samples, and the levels of LC3 protein expressed in these two tissues were measured. The study was approved by Qiqihar University and was conducted according to animal ethics guidelines and approved protocols (QDLISC-202303007).

Western blotting
Proteins from drug- or siRNA-treated cells in six-well cultures were extracted using radioimmunoprecipitation assay lysis buffer with protease inhibitor PMSF (Beyotime Co., Ltd., Nanjing, China). Lysed cells were centrifuged for 10 min at 12,000 g and 4 °C, and the supernatant was collected for analysis. For the western blot analysis, lysate proteins (30–50 μg) were resolved using 15% SDS-PAGE and transferred onto nitrocellulose transfer membranes. Membranes were blocked with 1% BSA (Sigma-Aldrich Corporation, St. Louis, MO, USA).
for 2 h at 37 °C and incubated with primary antibody for 2 h at room temperature with rabbit anti-LC3B, anti-p62 and mouse anti-β-actin (Sigma-Aldrich Corporation, St. Louis, MO, USA). Membranes were treated with IRDye 800 CW goat anti-mouse IgG or goat anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE, USA) as secondary antibodies for 1 h at room temperature. For detection, an Odyssey Infrared Fluorescence Scanning Imaging System (LI-COR Biosciences, Lincoln, NE, USA) was used.

**Laser confocal microscopy**

For detection of autophagosomes, 293T or C2C12 cells were transfected with 2.5 μg GFP-LC3 plasmid. When cells were 70–80% confluent in 20 mm-diameter culture dishes, and 24 h after treatment with 0.5 mg/mL of atropine, they were washed with PBS, fixed with absolute ethanol for 30 min, and the cell nuclei were stained with DAPI (Beyotime Biotechnology Co., Ltd., Nanjing, China). Green fluorescence dots of GFP-LC3 were observed by confocal laser with a Leica SP2 confocal system (Leica Microsystems).

**Transmission electron microscopy**

Transmission electron microscopy was used to investigate the autophagy method, and it was conducted as per existing methods; 293T cells in 25 cm² flasks were treated with atropine for 24 h, while mock cells were used as the control [19]. Ultrathin section images were viewed on an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan). Small single- or double-membrane vesicles, 0.3–2.0 μm in diameter, observed in the cytoplasm were categorized as autophagosome-like vesicles.

**Statistical analysis**

All experiments were performed at least three times, and the data are presented as the mean ± SD. The statistical data analysis was performed using one-way ANOVA. P < 0.05 was considered to represent a statistically significant difference.

**Results**

![Figure 1 Autophagy is triggered by atropine in 293T cells.](image)

Figure 1 Autophagy is triggered by atropine in 293T cells. (a) Western blot analysis of the expression of LC3II and p62 when treated with different drugs and intensity of bands: ratio of LC3II to LC3I. 1–7 indicates mock, adrenaline, insulin, atropine, furosemide, neostigmine posterior respectively. **P < 0.01 (vs. atropine group), P < 0.05 (vs. posterior group).** (b) Confocal laser observation of GFP-LC3 fluorescence dot aggregation after atropine has acted on 293T cells. Bar: 8 μm. (c) 293T cells treated with atropine for 36 h were fixed and subjected to TEM. Vesicles with the characteristics of autophagosome-like structures are indicated by black arrows in the cytoplasm. Bar: 1 μm. (d) Western blot analysis of the expression of LC3II and p62 when treated with different concentrations of atropine and intensity of bands: ratio of LC3II to LC3I. Shown are means ± SD from three independent experiments. **P < 0.01 (vs. 0.5 mg/mL group, 0.01 mg/mL group), ns P > 0.05 (vs. 0.005 mg/mL group, 0.05 mg/mL group).** ns indicates no significant difference. Statistical variance analysis was calculated using Student's t-test. LC3, microtubule-associated protein light chain.
Autophagy is triggered by atropine in C2C12 cells

C2C12 cells express nAChR, and do not express mAChR. In order to further analyze the autophagy effect of atropine-induced cells, C2C12 cells were treated by atropine. The results showed that the conversion of LC3II to LC3I in the cells treated with atropine was significantly higher than that in mock control groups. In addition, p62 expression was increased in atropine-treated C2C12 cells when compared to untreated cells. This suggested that atropine also induced incomplete autophagy in C2C12 cells (Figure 2a). To detect autophagosomes and confirm other observations, C2C12 cells were transfected with a GFP-LC3 plasmid that expressed a GFP-LC3 fusion protein green fluorescent and LC3 proteins for 24 h, and then treated with atropine for 24 h, with rapamycin as a positive control. In atropine-treated cells, GFP-LC3 proteins accumulated as green foci, while GFP-LC3 had a diffuse distribution in mock control cells (Figure 2b).

In atropine-treated C2C12 cells, several autophagic vesicles were observed through TEM. In contrast, autophagosome-like double or single membranes were rarely seen in the mock cells (Figure 2c). These results further show that atropine induces autophagy in C2C12 cells.

Subsequently, C2C12 cells were treated with different concentrations of atropine to further confirm its effect on autophagy. The results showed that atropine induced autophagosome formation in a dose-dependent manner within a certain concentration range. At concentrations less than 0.005 mg/mL, atropine no longer affected autophagy (Figure 2d).

Atropine does not induce autophagy in H9c2 cells

To explore whether the effect of atropine-induced autophagy depends on the antagonism of acetylcholine receptors, atropine treatment of H9c2 cells resulted in expressed mAChR, demonstrating that atropine did not cause the conversion of LC3II to LC3I in H9c2 cells (Figure 3a, 3b).

In order to clarify further the relationship between the effect of atropine on autophagy and mAChR, silencing of mAChR gene was undertaken using siRNA targeting m3AChR in H9c2 cells. siRNA-1 and siRNA-3 in the designed and synthesized three pairs of siRNAs significantly inhibited the expression of mAChR (Figure 3c). The results showed that atropine significantly induced autophagy in m3AChR-silenced cells (Figure 3d). Therefore, the effect of atropine on autophagy in vitro is independent of the antagonism of mAChR.

Effect of atropine on autophagy in vivo

In order to verify the effect of atropine in inducing autophagy in vivo, 7-week-old mice were intraperitoneally injected with atropine at a concentration of 0.01 mg/mL each day. After 14 days of treatment, the mice were necked and the hippocampus, heart, liver, tibialis anterior muscle, and smooth muscle were obtained to analyze the conversion of LC3II to LC3I and p62 expression. The results showed that autotipine did not change autophagy levels in the hippocampus (Figure 4a) and smooth muscle (Figure 4b), which express mAChR; in contrast, it induced the conversion of LC3II to LC3I and increased p62 expression in the liver, heart, and tibialis anterior, which do not express mAChR (Figure 4c). This further revealed that atropine-induced autophagy acts on other pathways rather than antagonizing mAChR.

Discussion

Autophagy is widely involved in various physiological activities, such as metabolism and immune response, and plays a dual role in the occurrence and development of various neurodegenerative diseases (such as Alzheimer's, Huntington's and Parkinson's); furthermore, it may play a protective role in the body under certain conditions [20]. It also accelerates the deterioration of these diseases to varying degrees [21]. Similarly, many different physiological processes also regulate the formation of autophagy [22, 23]. In this study, 293T cells were treated with different physiological regulators, because it was found that atropine significantly induced the conversion of LC3II to LC3I, it can be speculated that atropine may induce autophagy. Atropine has a rapid onset of action and short half-life, and can be

Figure 2 Autophagy is triggered by atropine in C2C12 cells. (a) Western blot analysis of the expression of LC3II and p62 when treated with atropine and intensity of bands: ratio of LC3II to LC3I, *P < 0.01 (vs. atropine group), ns P > 0.05 (vs. PBS group). (b) Confocal laser observation of GFP-LC3 fluorescence dot aggregation after atropine has acted on C2C12 cells. Bar: 8 μm. (c) C2C12 cells treated with atropine for 36 h were fixed and subjected to TEM. Vesicles with the characteristics of autophagosome-like structures are indicated by black arrows in the cytoplasm. Bar: 1 μm. (d) Western blot analysis of the expression of LC3II and p62 when treated with different concentrations of atropine and intensity of bands: ratio of LC3II to LC3I. Shown are means ± SD from three independent experiments. *P < 0.01 (vs. 0.5 mg/mL group, 0.01 mg/mL group), ns P > 0.05 (vs. 0.005 mg/mL group, 0.05 mg/mL group), ns indicates no significant difference. Statistical variance analysis was calculated using Student's t-test. PBS, phosphate-buffered saline; LC3, microtubule-associated protein light chain.
Figure 3 The effect of rapamycin and starvation induction and atropine on autophagy in H9c2 cells. (a) Western blot analysis of the expression of LC3II and p62 when treated with atropine and intensity of bands ratio of LC3II to LC3I. *P < 0.01 (vs. rapamycin group), **P < 0.01 (vs. starvation group), ns (vs. atropine group). (b) Western blot analysis of the expression of LC3II and p62 when treated with different concentrations of atropine. (c) Silencing effect of siRNAs on m3AChR. (d) Effects of atropine on autophagy after silencing m3AChR. Shown are means ± SD from three independent experiments, ns P > 0.05 (vs. PBS group), ns P > 0.05 (vs. siNC group), *P < 0.01 (vs. siRNA group). ns indicates no significant difference. Statistical variance analysis was calculated using Student’s t-test. PBS, phosphate-buffered saline; LC3, microtubule-associated protein light chain.

Figure 4 The effect of intraperitoneal injection of atropine on autophagy in mice. (a) Western blot analysis of the expression of LC3II and p62 in the hippocampus of mice injected with atropine, acetylcholine, and huperzine A as controls. (b) Western blot analysis of the expression of LC3II and p62 in the smooth muscle of mice injected with atropine. (c) Western blot analysis of the expression of LC3II and p62 in the liver, heart, tibialis anterior of mice injected with atropine. Shown are means ± SD from three independent experiments. Statistical variance analysis was calculated using Student’s t-test. PBS, phosphate-buffered saline; LC3, microtubule-associated protein light chain.

used during anesthesia for parenteral rescue, including cardiac arrhythmias, and for prevention of vagal reflexes, reduction of secretions, acute bronchospasms, and anticholinesterase overdose or poisoning [7, 8, 24]. However, no other effects of atropine other than as an antagonist of mAChR have been found. Karla et al. found that immunization with plasmids encoding M2 acetylcholine muscarinic receptor epitopes impairs cardiac function in mice and induces autophagy in the myocardium [25]. Zhao et al. reported that mAChRs were involved in acetylcholine-induced autophagy to provide protection for cardiomyocytes during cardiomyocyte hypoxia/reoxygenation injury, and that mAChRs promoted Ach-induced autophagy via AMPK-mTOR-dependent pathway [26]. In this study, atropine induced autophagosome levels in a dose-dependent manner within a certain concentration range above 0.005 mg/mL, below which it no longer affected autophagy. In atropine-treated cells, GFP-LC3 proteins accumulated as green foci, whereas GFP-LC3 had a diffuse distribution in mock control cells. These results further show that atropine induces autophagy in 293T cells. Since 293T cells do not contain active acetylcholine receptors, the effect of atropine on autophagy in 293T cells may not be dependent on the action of mAChR. It was found that atropine induced autophagy in C2C12 cells but not in H9c2 cells, which contain nAChR and mAChR, respectively. Therefore, in this study, the effect of atropine treatment on autophagy
in vitro was not dependent on the inhibition of mAChR. Similarly, in vivo experiments also showed that atropine did not induce autophagy in tissue cells which expressed mAChR.

In mAChR-expressing cells, atropine preferentially binds to mAChR but does not induce subsequent responses to autophagy, and its inability to bind to other related receptors also results in the inability to induce autophagy. In this study, RNA-seq analysis was performed on atropine treated 293T cells to investigate the possible role of atropine receptors in inducing autophagy. The results showed that the expression level of Rag gene was significantly increased. When considering that Rag plays a critical role in amino acid-dependent mTORC1 activation by targeting mTORC1 to lysosomes, Rag GTPase provides an important clue regarding the mechanism underlying autophagy induction in response to amino acids [27, 28]. However, the specific molecular mechanism still requires further elucidation.

References
