Tiotropium/Olodaterol Reduce Cigarette Smoking Extract-induced Cell Death in BEAS-2B Bronchial Epithelial Cells

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Abstract

Background COPD is characterized by progressive irreversible airflow limitation. Cigarette smoking is an important risk factor for destruction of lung parenchyma or emphysema. Disruption of epithelial layer integrity may contribute to lung injury in response to CS exposure. Tiotropium/Olodaterol act as bronchodilator to treatment COPD by preventing ASM contraction and muscle relaxation; however, the effect of dual bronchodilator on epithelial cell injury and its underlying mechanism is still unclear. In this study, we aim to evaluate the effect of Tiotropium/Olodaterol on CSE-mediate cell death, with a special focus on autophagy.

Methods In this study, we investigated the effects of Tiotropium/Olodaterol on cell viability, the mitochondrial membrane potential (MMP; Δφm), and autophagy in BEAS-2B bronchial epithelial cells.

Result: Tiotropium/olodaterol significantly inhibit CSE-induce cell death, mitochondria dysfunction and autophagy in response to short-term treatment with CSE. Moreover, decrease in ERK and JNK activation after CSE treatment were reversed by Tiotropium/olodaterol treatment.

Conclusion: Tiotropium/olodaterol have protective effect against CSE-induced cell death which may related to induction of autophagy via activating ERK and JNK. In this study, we provide a possible novel mechanism for dual bronchodilator in COPD treatment.

Background

COPD is an abnormal persist inflammatory disease characterized by progressive irreversible airflow limitation [1]. Cigarette smoking is an important risk factor for advancing inflammation in airway and destruction of lung parenchyma or emphysema [2]. Previous studies have reported that smokers have faster FEV_1 decline compare with nom-smoker. Moreover, the severity of COPD is correlated with the amount of cigarette smoking [3]. In recent, cigarette smoking is known to induce cell death in lung structure cell which contribute to development of pulmonary emphysema in the lungs of smoker have been documented [4, 5]. Therefore, the underlying mechanism of CSE-induce cell death is an active work area, and the information from which suggest that cell death play crucial roles in emphysema and COPD. Epithelial cell forms an essential barrier for preserving the airway function. To be a major airway first-line defense system, the bronchial epithelium is inevitable to undergo CS exposure. Disruption of epithelial layer integrity may generate some of earliest elements of lung injury in response to CS exposure such as ROS [6, 7]. Moreover, several studies demonstrated that programmed cell death including apoptosis and autophagy were involved in CS-induced epithelial cell injury [8, 9]. Thus, inhibition of CS-induce cell death may have a therapeutic strategy in COPD progression.

Autophagy is a highly conserved self-degradative process in eukaryotes which balance sources of energy in many physiological conditions. In addition, autophagy is also a self-degradative process with functions including the removal of misfolded or aggregated proteins, and damaged organelles [10]. Recently, several evidences suggest that increased autophagy contributes to COPD pathogenesis. In vitro

studies demonstrated that autophagy activation contribute to CS-induced cilia shortening and mitochondrial dysfunction in airway epithelium as well [11, 12]. Moreover, cigarette smoke induced epithelial cell through activating autophagy via modulating various signaling pathway were identified [9, 10, 13, 14]. These findings suggest that autophagy is a deleterious process by regulating various damage processes in airway epithelium during COPD pathogenesis.

According to the Global Initiative for COPD (GOLD) recommendation, bronchodilators are currently the mainstay of pharmacotherapy for COPD. The use of dual bronchodilator with a long-acting muscarinic antagonist (LAMA) plus a long-acting β2-agonist (LABA) have been considered when symptoms are not improved with monotherapy. Inhibition of muscarinic receptor prevent airway smooth muscle (ASM) contraction, while activation of β2-adrenoceptors induces muscle relaxation, thus producing a bronchodilator effect [15]. The rational for combining two bronchodilator withe different duration and mechanism of action would be to increase synergistic bronchodilator effect reduce the overall risk of adverse event and avoid drugs with overlapping toxicities [16]. Tiotropium/Olodaterol is LAMA/LABA inhalation spray which is approved in several countries for the long-term maintenance treatment of COPD. Tiotropium/Olodaterol improve lung function to a greater extent than monotherapy have document. Moreover, Tiotropium/Olodaterol has beneficial effect on dyspnoea inspiratory capacity and HR-QoL [17].

In previous study, M3R and β2AR were involved in the mechanism of cell death. For instance, β2-Aabs levels may be associated with worse alveolar airspace destruction and aggravate smoking related lung injuries have been documented [18]. Furthermore, M3 mAChR activation inhibit cell proliferation have been found in previous study [19]. These evidences suggest that regulation of muscarinic receptor and β2AR may be a protection strategy against cell death. Tiotropium/Olodaterol as dual bronchodilator is reported to reduce ROS generation, which were indicated as a cell death inducer [20]. However, the role of dual bronchodilator on CSE-induced cell and its underlying mechanism is still unclear. In this study, we hypothesis dual bronchodilator may contribute to protect epithelial cell from death by regulating autophagy. We aim to evaluate the effect of Tiotropium/Olodaterol on CSE-mediate cell death, with a special focus on autophagy.

Methods

Chemicals and reagents

Tiotropium and Olodaterol were purchased from Tocris (Ellisville, MO, USA). Acridine orange (N,N,N',N'-Tetramethylacridine-3,6-diamine), 5,5’,6,6’-tetrachloro-1,10,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Sigma-Aldrich, St. Louis, MO, USA) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from (St. Louis, MO, USA). All of these chemicals were dissolved in DMSO and stored in -20℃. MTT, JC-1 and acridine orange were dissolved in water and stored in 4℃.

Antibodies

Anti-LC-3 and Anti-Beclin 1were purchased from Novus Biological (Littleton, CO, USA). Anti-Phospho-ERK and Anti-Phospho-JNK were purchased from Santa Cruz. (Santa Cruz, CA, USA). GAPDH was purchased from Cell signaling (Danvers, MA, USA). Goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson Laboratory (Bar Harbor, ME, USA).

Cell culture

BEAS-2B human lung epithelial cell lines were kindly provided by Dr. Chi-Chien Lin (Department of Life Sciences, Institute of Biomedical Science, National Chung Hsing University, Taiwan.). The BEAS-2B human lung epithelial cells were culture in RPMI 1640 medium containing 10% fetal bovine serum (FBS; Gibco,Gland Island, NY, USA) and 1% antibiotic antimycotic. Cells maintain in an incubator with a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells were grown to 90% confluence and passaged by trypsin/EDTA.

Preparation of cigarette smoking extraction

Commercial cigarettes (LONG LIFE, Taiwan), which contains 1.2 mg of nicotine and 12 mg of tar, were used for cigarette smoking extraction (CSE) preparation. A five cigarettes smoke was bubbled through 10 ml of PBS. The solution was regarded as 100% strength CSE. The peristaltic pump was equilibrated at a rate of one cigarette per 5 min. Then the CSE suspension was filtered through a 0.22-μm-pore filter to remove bacteria and particles.

Cell viability assay

BEAS-2B cells were seeded in 24-well plates at a density of 2×10^4 cell /ml and pre-treated with and without various concentrations of Tiotropium/Olodaterol for 4 h. Then, cells were cultured with 0–10% CSE for 24 h. After 24 h incubation, 200 μl of 1 mg/ml MTT in RPMI 1640 was added to each well 4 h before the end of each incubation. The MTT solution was removed after 4 h. The adherent cells were lysed with 600 μl DMSO and the optical density (OD) was obtained at 570 nm using a microplate reader (PerkinElmer).

Detection of the mitochondrial membrane potential

BEAS-2B cells were seeded in 6-well plates at a density of 2 × 105 cell /ml and pre-treated with and without various concentrations of Tiotropium/Olodaterol for 4 h. Then, cells were cultured with 0–10% CSE for 6. After incubation, cells were removed from the plate with trypsin-EDTA (GIBCO-BRL). 10 μg/mL 5,5',6,6'-tetrachloro-1,10,3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Sigma-Aldrich, St. Louis, MO, USA) and serum free medium were mixed in the ratio of 1:500, and 1 ml of the mixture was added to each sample followed by incubation at 37℃ for 15 min. Sample were detected and quantified by FACS.

Acridine orange assay

Autophagy is characterized by the formation of acidic vesicular organelles (AVOs). We used acridine orange for detecting the acidic cellular compartment which emits bright red fluorescence in acidic vesicles while fluoresces green in the cytoplasm and nucleus. BEAS-2B cells were seeded in 6-well plates

at a density of 2×10^5 cell /ml and pre-treated with and without various concentrations of Tiotropium/Olodaterol for 4 h. Then, cells were cultured with 0–10% CSE for 6 and 24 h. After incubation, cell were removed from the plate with trypsin-EDTA (GIBCO-BRL) acridine orange was then added at a final concentration of 1 mg/mL and incubate at 37 °C for a period of 15 min. Then, the AVO in BEAS-2B cells were detected and quantified by FACS. AVO showed bright red fluorescence (650 nm, FL-3 channel) whereas cytoplasm and nucleolus showed green fluorescence (500–550 nm, FL-1 channel) [18]. Intensity of red fluorescence is proportional to number of AVO in autophagic cells.

Western blot

Cells were lysed in RIPA buffer with 10% proteasome inhibitor and cells extracts were cleared at 12000 rpm in a microcentrifuge at 4°C for 20 min. Proteins were separated by 10 and 15% SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST) buffer and then immunostaining was performed with anti-LC-3, anti-Beclin 1, anti-phospho-ERK, anti-phospho-JNK, followed by HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies. ECL reagent (GE Healthcare Life Sciences, Chalfont, UK),) was used for protein detection.

Statistical analysis

All values are expressed as the mean ± SD. For comparison between two groups, we used unpaired two-tailed t test (Student's t test) and values of $p < 0.05$ were considered statistically significant. The data were analyzed with GraphPad Prism Version 6.0 (San Diego, CA, USA).

Results

Cigarette smoking extract induce cell death in BEAS-2B bronchial epithelia cells

To evaluate the effect of cigarette smoking extract on BEAS-2B bronchial epithelia cells. BEAS-2B cells were exposure to various dosage of CSE. **As shown in** Fig. 1. CSE treatment significantly reduce cell viability in 5% CSE and 10% at 24 hours treatment. And the IC50 were approximately 5% CSE. The dose lower than 2.5% CSE show slight toxicity, while the dose grater that 2.5%. These results show that CSE treatment remarkably increase bronchial cell injury in dose greater than 5% CSE.

Tiotropium/olodaterol reverse CSE-induced cell death in BEAS-2B bronchial epithelia cells

To evaluate the effect of tiotropium/olodaterol on CSE-induced cell death. We pre-treat BD for 4 hours followed by 5% CSE treatment for 24 hours and the cell viability were determined by MTT assay. **As shown in** Fig. 2, pre-treatment with BD were significantly increase in cell viability compared with 5% CSE treatment alone. This result show that pre-treatment with BD have protective effect against cell injury caused by CSE treatment.

Tiotropium/olodaterol reverse CSE-induced mitochondria membrane potential disruption in BEAS-2B bronchial epithelia cells

It is established that changes in mitochondrial membrane potential (Δψm) are integral to the cell life/death transition. To evaluated whether CSE affected the mitochondrial membrane potential (Δψm) and the effect of Tiotropium/olodaterol on CSE-mediated MMP change. **As shown in** Fig. 3, treated cells were stained with the fluorescent cationic dye JC-1. Loss of Δψm is an indicator of mitochondrial damage during cell death. After cells were treated with 5% CSE for 24 h, red fluorescence was detected in BEAS-2B cells, suggesting a reduction in Δψm caused by 5% CSE. Furthermore, pre-treatment with Tiotropium/olodaterol decrease the red fluorescence expression. BEAS-2B cells were pretreated 4 h with Tiotropium/Olodactoro and then treated with 5% CSE for 24 h showed significantly retard mitochondrial dysfunction.

Tiotropium/olodaterol inhibit CSE-induced autophagy in BEAS-2B bronchial epithelia cells

Previous studies indicated that autophagy is an important pathway of programmed cell death as well as COPD pathogenesis [4, 5]. We evaluated whether 5% CSE induce autophagy and the effect of pretreatment with Tiotropium/olodaterol. We firstly evaluated autophagy by acridine orange staining (AO) in FACS analysis. For autophagolysosomes, the protonated form of AO accumulates and aggregates, which are characterized by yellow-orange fluorescence (FL3). Staining of normal cells with AO, a weak base, which is characterized by green fluorescence (FL1). **As shown in** Fig. 4A, CSE treatment resulted in the appearance of yellow-orange (FL-3) fluorescence after 24 h. Another autophagy marker, named the conversion of LC3-I (18 kD) to LC3-II (16 kD), were analysed by western blot.. **As shown in** Fig. 4B, CSE treatment for 24 h, the ratio of LC-II/LC3-I as well as LC3-II level were increased in BEAS-2B bronchial epithelial cells. Pre-treatment with Tiotropium/olodaterol for 4 h, AVO and the conversion of LC3-I to LC3-II slightly decrease in compared with 5% CSE treatment only.

Notably, CSE treatment resulted in the appearance of yellow-orange (FL-3) fluorescence were also found in 6 h **(**Fig. 5A**)**. Furthermore, CSE treatment for 6 h, the ratio of LC-II/LC3-I as well as LC3-II level were increased in BEAS-2B bronchial epithelial cells **(**Fig. 5B**)**. Pre-treatment with Tiotropium/olodaterol for 4 h, AVO and the conversion of LC3-I to LC3-II significantly decrease in compared with 5% CSE treatment only. These finding suggest that Tiotropium/olodaterol have inhibitory effect on autophagy in short-term of CSE treatment.

Tiotropium/olodaterol induce ERK and JNK activation in BEAS-2B bronchial epithelia cells

ERK and JNK activation were involved in cell survival and cell death by regulating autophagy [21, 22]. To further understand the molecular mechanisms of action of Tiotropium/olodaterol on the underlying mechanism. We examined the effect of Tiotropium/olodaterol on ERK and JNK. **As shown in** Fig. 6A and 6B, pre-treatment with Tiotropium/olodaterol significantly increase ERK activation as well as JNK, following by CSE treatment for 3 h and 6r, respectively. These findings suggest that Tiotropium/olodaterol inhibit autophagy via inducing ERK and JNK activation.

Discussion

Tiotropium and olodaterol combination is used as long-term maintenance treatment in COPD patients, including chronic bronchitis or emphysema [16]. It act as bronchodilator by preventing ASM contraction and inducing muscle relaxation by regulating muscarinic receptor and β2 beta2 adrenergic receptor, respectively [15]. In recent, there are few studies indicate that bronchodilator have protective effect in CSE-induced lung epithelia cell injury by reducing inflammatory response [24, 25]. However, the effect of dual-bronchodilator on CSE-induced cell injury in epithelia cell is still elusive. In current study, we have evaluated the protective effect of Tiotropium/olodaterol in BEAS-2B bronchial epithelial cells by reducing cell death and autophagy through activating ERK and JNK pathway, which induced by CSE. These findings provide a novel evidence about new action of dual-bronchodilator against CSE-induced lung epithelial cell injury, which may be more associated with human COPD as bronchial epithelial cells play a critical role in disease pathogenesis.

Cigarette smoking is the well-known major risk factor of COPD. Therefore, cigarette smoking extract is logical choice to experimentally explore the mechanism and pathogenesis of COPD in vitro. Moreover, there are accumulative evidence indicated that cigarette smoking-induce lung injury is related to epithelial cell disruption [6, 8, 9,12]. In present study, we also demonstrate CSE treatment cause cell death in lung epithelial cell. Our in vitro study indicated CSE treatment significantly reduced BEAS-2B bronchial epithelial cells survival, and that dramatically increased autophagy and mitochondria damage were the crucial contributor to CSE-induced cell death. Notably, CSE-induced cell death were obviously reversed by Tiotropium/olodaterol treatment. To our best knowledge, this is the first study to evidence that Tiotropium/olodaterol have protective effect against CSE-induced cell death. These finding suggest that increase survival of lung epithelial cell may be an additional effect of Tiotropium/olodaterol for COPD pharmacotherapy.

Besides oxidative stress, inflammation, apoptosis, and imbalance of protease-antiprotease, autophagy is regarded as the important contributor to the development of COPD have been documented in recent [25]. Moreover, various studies found that inhibition of autophagy controls lung cell death and the emphysema development in mice caused by CS exposure [4, 5]. It suggest that autophagy may be a critical target for regulating cigarette smoke induced lung injury. Although there are few studies report the action of bronchodilator on modulating autophagy in lung epithelial cell. Pervious study have shown that activation of β-arrestin2, the downstream of β2 beta2 adrenergic receptor, contribute to inhibit autophagy lead to decrease in inflammation in BEAS-2B cell have been reported [26]. Moreover, the muscarinic receptor antagonist atropine decreased ACh-induced autophagy were also indicated [27]. These evidence suggest that autophagy may be important regulatory for dual-bronchodilator in modulating epithelial cell death. Our finding show that Tiotropium/olodaterol treatment decreased LC-3I concert to LC-3II as well as Beclin1 and also reduce AVO expression, the marker of autophagy activation. Although the inhibitory effect of Tiotropium/olodaterol on autophagy in long-term of CSE exposure (24h) is not significant, while Tiotropium/olodaterol treatment dramatically ameliorate CES-induced autophagy in short-term (6h). The finding suggest that inhibition of autophagy by Tiotropium/olodaterol treatment may act as adaptive response against cell death which contribute to protect epithelial cell in response to CSE stimulation.

ERK and JNK signaling pathway were involved in cell survival and cell death by regulating autophagy [21, 22]. However, the interactions between autophagy and activation of ERK are complex as well as JNK. ATG5, autophagy-related protein, serve as cellular scaffolds to induce ERK phosphorylation [28]. Inhibition of JNK expression decrease Beclin 1 and represses autophagy [29]. On the other hand, sustained ERK activation may suppression the autophagic flux and MAPK/JNK activation prevent induction of autophagy via activating mTOR have been reported [30]. In our study, we found that Tiotropium/olodaterol significantly induced ERK and JNK activation after CSE treatment which is in contrast with the trend of autophagy. These finding suggest that activation of ERK and JNK signaling pathway may contribute to prevent CSE-induced cell death by inhibiting autophagy in BEAS-2B bronchial epithelial cells.

Conclusions

We demonstrate that Tiotropium/olodaterol reverse CSE-induced cell death in BEAS-2B bronchial epithelial cells. To our best knowledge, this is the first study to evaluate the effect of dual bronchodilator on CSE-induced cell death and its underlying mechanism. Tiotropium/olodaterol significantly inhibit CSE-induce cell death, mitochondria dysfunction and autophagy in short-term treatment with CSE. Moreover, decrease in ERK and JNK activation after CSE treatment were reversed by Tiotropium/olodaterol treatment. Current findings suggest Tiotropium/olodaterol have protective effect against CSE-induced cell death which may related to induction of autophagy via activating ERK and JNK. In this study, we provide a possible novel mechanism for dual-bronchodilator in COPD treatment.

Abbreviations

COPD
chronic obstructive pulmonary disease
CSE
cigarette smoking extraction
ERK
extracellular-signal-regulated kinase
JNK
c-Jun N-terminal kinase
FEV1
forced expiratory volume in one second
ROS
reactive oxygen species
LAMA
long acting muscarinic antagonist
LABA
long acting β2 sympathomimetic agonists

mAChR
muscarinic acetylcholine receptor
β2AR
β2-adrenergic receptor
M3R
M3 muscarinic acetylcholine receptor

Declarations

Competing interests

The authors declare that they have no competing interests regarding this study.

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Authors' contributions

C.-H.C, Y.-R.L. and C.-H.L. conceived and designed the experiments. S.-H.L., H.-H.C.,. performed the experiments. W.-H.C. and P.-C.C. analyzed the data. Y.-R.L., and C.-H.L. wrote the manuscript. All authors read and approved the final manuscript.

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Not applicable.

References

1. Agustí A, Hogg JC. Update on the Pathogenesis of Chronic Obstructive Pulmonary Disease. N Engl J Med. 2019 Sep;26(13):1248–56. 381(.
2. Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2001 May;163(6):1304–9.
3. Liu Y, Pleasants RA, Croft JB, Wheaton AG, Heidari K, Malarcher AM, Ohar JA, Kraft M, Mannino DM, Strange C. Smoking duration, respiratory symptoms, and COPD in adults aged ≥ 45 years with a smoking history. Int J Chron Obstruct Pulmon Dis. 2015 Jul 21;10:1409-16.
4. Chen ZH, Kim HP, Sciurba FC, Lee SJ, Feghali-Bostwick C, Stolz DB, Dhir R, Landreneau RJ, Schuchert MJ, Yousem SA, Nakahira K, Pilewski JM, Lee JS, Zhang Y, Ryter SW, Choi AM. Egr-1 regulates

autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. PLoS One. 2008 Oct 2;3(10):e3316.

5. Chen ZH, Lam HC, Jin Y, Kim HP, Cao J, Lee SJ, Ifedigbo E, Parameswaran H, Ryter SW, Choi AM. Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema. Proc Natl Acad Sci U S A. 2010 Nov 2;107(44):18880-5.
6. Miao Q, Xu Y, Zhang H, Xu P, Ye J. Cigarette smoke induces ROS mediated autophagy impairment in human corneal epithelial cells. Environ Pollut. 2019 Feb;245:389–97.
7. Lin XX, Yang XF, Jiang JX, Zhang SJ, Guan Y, Liu YN, Sun YH, Xie QM. Cigarette smoke extract-induced BEAS-2B cell apoptosis and anti-oxidative Nrf-2 up-regulation are mediated by ROS-stimulated p38 activation. Toxicol Mech Methods. 2014 Dec;24(8):575–83.
8. Vanella L, Li Volti G, Distefano A, Raffaele M, Zingales V, Avola R, Tibullo D, Barbagallo I. A new antioxidant formulation reduces the apoptotic and damaging effect of cigarette smoke extract on human bronchial epithelial cells. Eur Rev Med Pharmacol Sci. 2017 Dec;21(23):5478–84.
9. Wu D, Yuan Y, Lin Z, Lai T, Chen M, Li W, Lv Q, Yuan B, Li D, Wu B. Cigarette smoke extract induces placental growth factor release from human bronchial epithelial cells via ROS/MAPK (ERK-1/2)/Egr-1 axis. Int J Chron Obstruct Pulmon Dis. 2016 Dec 2;11:3031–3042.
10. Ravanan P, Srikumar IF, Talwar P. Autophagy: The spotlight for cellular stress responses. Life Sci. 2017 Nov 1;188:53–67.
11. Lam HC, Cloonan SM, Bhashyam AR, Haspel JA, Singh A, Sathirapongsasuti JF, Cervo M, Yao H, Chung AL, Mizumura K, An CH, Shan B, Franks JM, Haley KJ, Owen CA, Tesfaigzi Y, Washko GR, Quackenbush J, Silverman EK, Rahman I, Kim HP, Mahmood A, Biswal SS, Ryter SW, Choi AM. Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction. J Clin Invest. 2013 Dec;123(12):5212–30.
12. Sundar IK, Maremanda KP, Rahman I. Mitochondrial dysfunction is associated with Miro1 reduction in lung epithelial cells by cigarette smoke. Toxicol Lett. 2019 Dec 15;317:92–101.
13. Wu Y, Li Y, Wu B, Tan C, He X, Xu B, Yu G, Wang H. β-Arrestin2 Inhibits Expression of Inflammatory Cytokines in BEAS-2B Lung Epithelial Cells Treated with Cigarette Smoke Condensate via Inhibition of Autophagy. Cell Physiol Biochem. 2018;50(4):1270–85.
14. Wang Y, Liu J, Zhou JS, Huang HQ, Li ZY, Xu XC, Lai TW, Hu Y, Zhou HB, Chen HP, Ying SM, Li W, Shen HH, Chen ZH. MTOR Suppresses Cigarette Smoke-Induced Epithelial Cell Death and Airway Inflammation in Chronic Obstructive Pulmonary Disease. J Immunol. 2018 Apr 15;200(8):2571–2580.
15. Cazzola M, Rogliani P, Matera MG. The future of bronchodilation: looking for new classes of bronchodilators. Eur Respir Rev. 2019 Dec 23;28(154). pii: 190095.
16. Blair HA. Tiotropium/Olodaterol: A Review in COPD. Drugs. 2019 Jun;79(9):997–1008.
17. Calzetta L, Matera MG, Rogliani P, Cazzola M. Dual LABA/LAMA bronchodilators in chronic obstructive pulmonary disease: why, when, and how. Expert Rev Respir Med. 2018 Apr;12(4):261–4.

18. Hu JY, Liu BB, Du YP, Zhang Y, Zhang YW, Zhang YY, Xu M, He B. Increased circulating β2-adrenergic receptor autoantibodies are associated with smoking-related emphysema. Sci Rep. 2017 Mar;6:7:43962.
19. Shafer SH, Williams CL. Elevated Rac1 activity changes the M3 muscarinic acetylcholine receptor-mediated inhibition of proliferation to induction of cell death. Mol Pharmacol. 2004 May;65(5):1080–91.
20. Profita M, Albano GD, Montalbano AM, Di Sano C, Anzalone G, Gagliardo R, Riccobono L, Bonanno A, Siena L, Pieper MP, Gjomarkaj M. Acetylcholine leads to signal transducer and activator of transcription 1 (STAT-1) mediated oxidative/nitrosative stress in human bronchial epithelial cell line. Biochim Biophys Acta. 2013 Dec;1832(12):1949–58.
21. Zhou YY, Li Y, Jiang WQ, Zhou LF. MAPK/JNK signalling: a potential autophagy regulation pathway. Biosci Rep. 2015 Apr 22;35(3). pii: e00199.
22. Corcelle E, Djerbi N, Mari M, Nebout M, Fiorini C, Fénichel P, Hofman P, Poujeol P, Mograbi B. Control of the autophagy maturation step by the MAPK ERK and p38: lessons from environmental carcinogens. Autophagy. 2007 Jan-Feb;3(1):57–9.
23. Toumpanakis D, Loverdos K, Tzouda V, Vassilakopoulou V, Litsiou E, Magkou C, Karavana V, Pieper M, Vassilakopoulos T. Tiotropium bromide exerts anti-inflammatory effects during resistive breathing, an experimental model of severe airway obstruction. Int J Chron Obstruct Pulmon Dis. 2017 Jul 28;12:2207–2220.
24. Kolahian S, Shahbazfar AA, Tayefi-Nasrabadi H, Keyhanmanesh R, Ansarin K, Ghasemi H, Rashidi AH, Gosens R, Hanifeh M. Tiotropium effects on airway inflammatory events in the cat as an animal model for acute cigarette smoke-induced lung inflammation. Exp Lung Res. 2014 Aug;40(6):272–87.
25. Fischer BM, Pavlisko E, Voynow JA. Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation. Int J Chron Obstruct Pulmon Dis. 2011;6:413–21.
26. Wu Y, Li Y, Wu B, Tan C, He X, Xu B, Yu G, Wang H. β-Arrestin2 Inhibits Expression of Inflammatory Cytokines in BEAS-2B Lung Epithelial Cells Treated with Cigarette Smoke Condensate via Inhibition of Autophagy. Cell Physiol Biochem. 2018;50(4):1270–85.
27. Zhao M, Sun L, Yu XJ, Miao Y, Liu JJ, Wang H, Ren J, Zang WJ. Acetylcholine mediates AMPK-dependent autophagic cytoprotection in H9c2 cells during hypoxia/reoxygenation injury. Cell Physiol Biochem. 2013;32(3):601–13.
28. Pattingre S, Bauvy C, Codogno P. Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. J Biol Chem. 2003;278:16667–74.
29. Wang J, Whiteman MW, Lian H, Wang G, Singh A, Huang D, Denmark T. A non-canonical MEK/ERK signaling pathway regulates autophagy via regulating Beclin 1. J Biol Chem. 2009 Aug 7;284(32):21412–24.
30. Basu S, Rajakaruna S, Reyes B, Van Bockstaele E, Menko AS. Suppression of MAPK/JNK-MTORC1 signaling leads to premature loss of organelles and nuclei by autophagy during terminal

differentiation of lens fiber cells. Autophagy. 2014 Jul;10(7):1193–211.

Figures

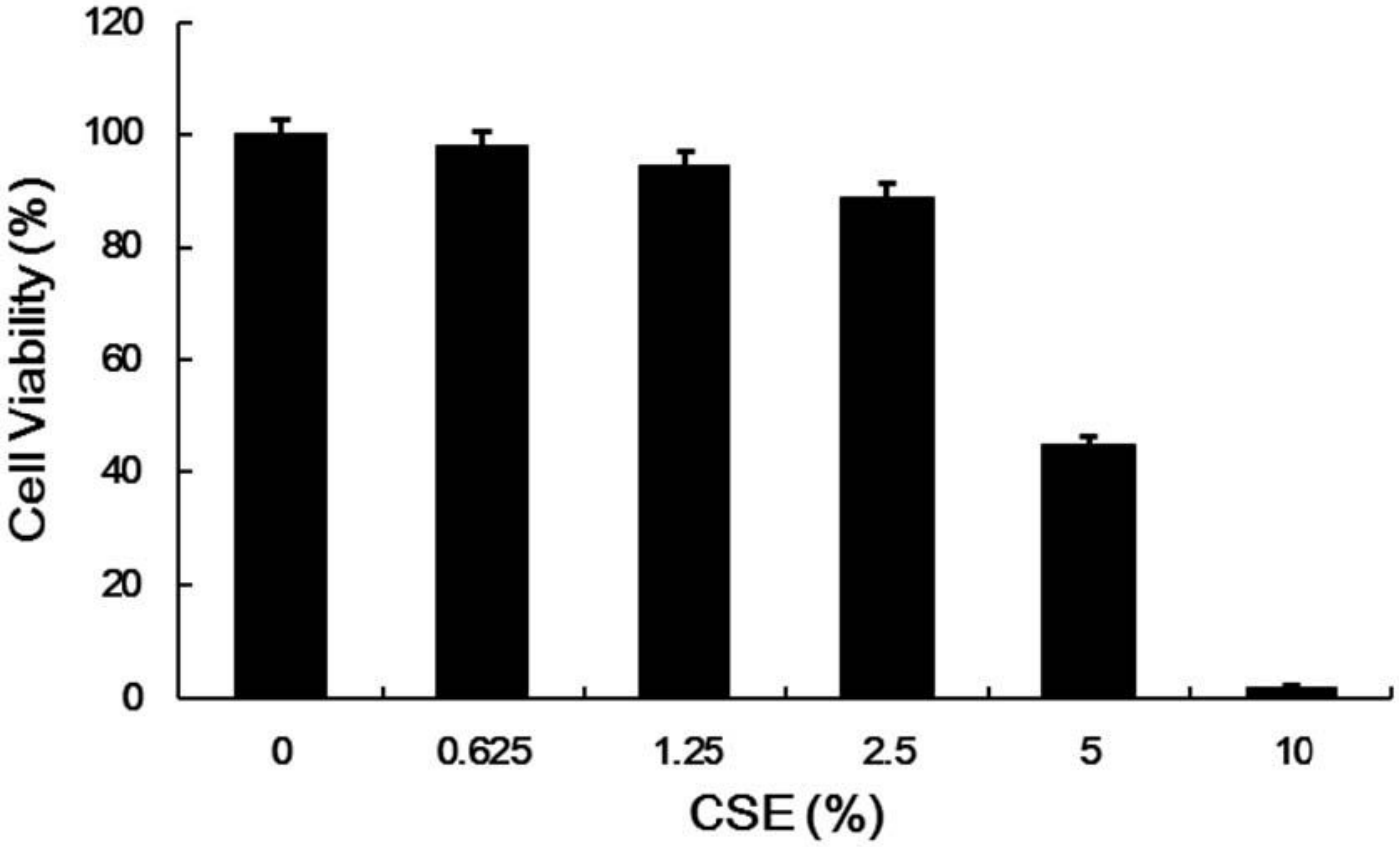

Figure 1

Effects of cigarette smoke extraction (CSE) on the viability of BEAS-2B cells. Cell viability of BEAS-2B cells after treatment with various concentrations of CSE for 24 h. Cell viability was determined by the MTT assay. The absorbance of the reaction solution at 570 nm was recorded. Data are presented as means ± SD from triplicate samples for each treatment.

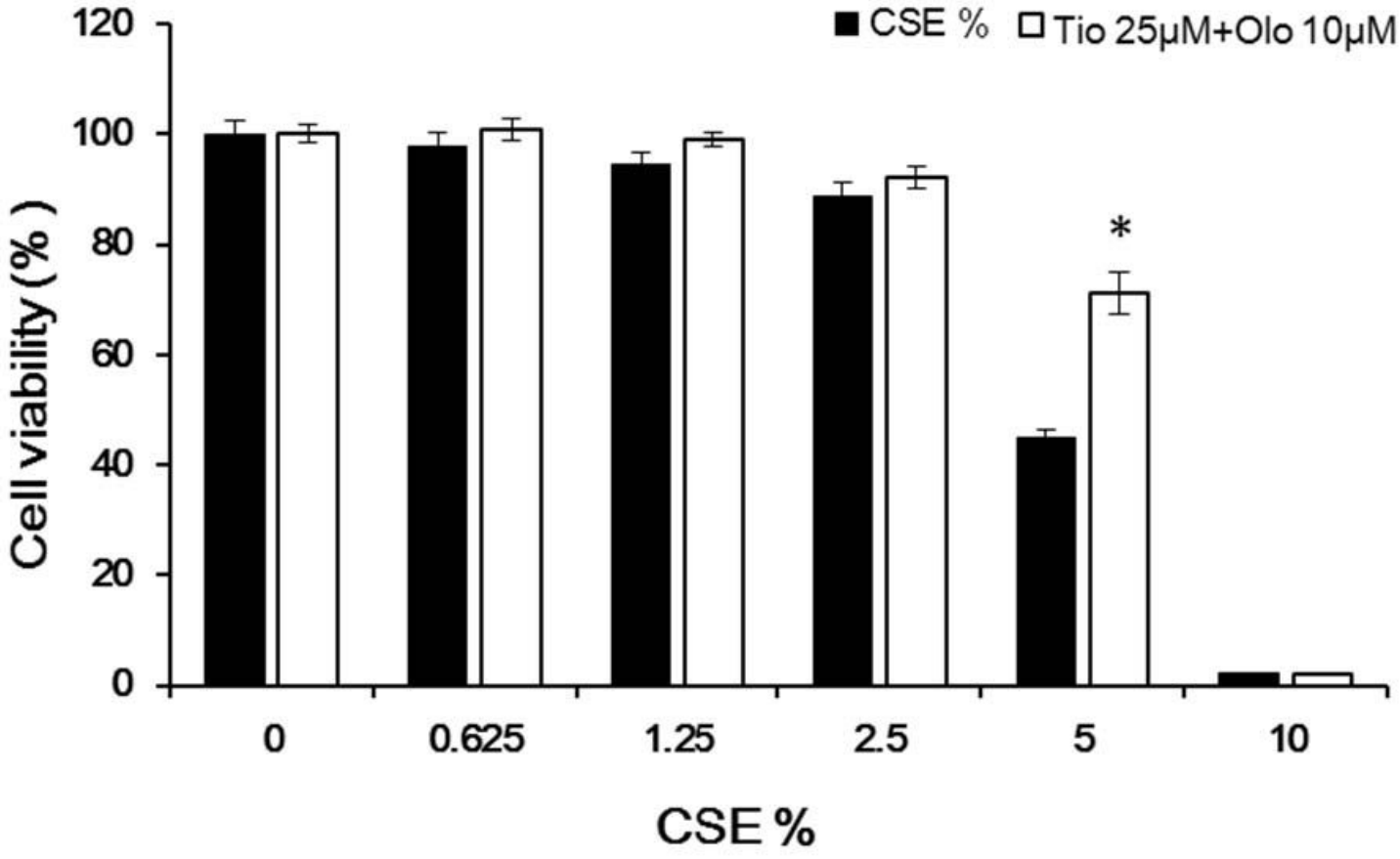

Figure 2

Effects of Tiotropium/Olodaterol on CSE-induced cell death in BEAS-2B cells. Cell viability of BEAS-2B cells after pre-treatment with 25μM tiotropium+10 μM Olodaterol for 4h, following by CSE treatment for 24 h. Cell viability was determined by the MTT assay. The absorbance of the reaction solution at 570 nm was recorded. Data are presented as means ± SD from triplicate samples for each treatment. Tio: tiotropium; Olo: Olodaterol

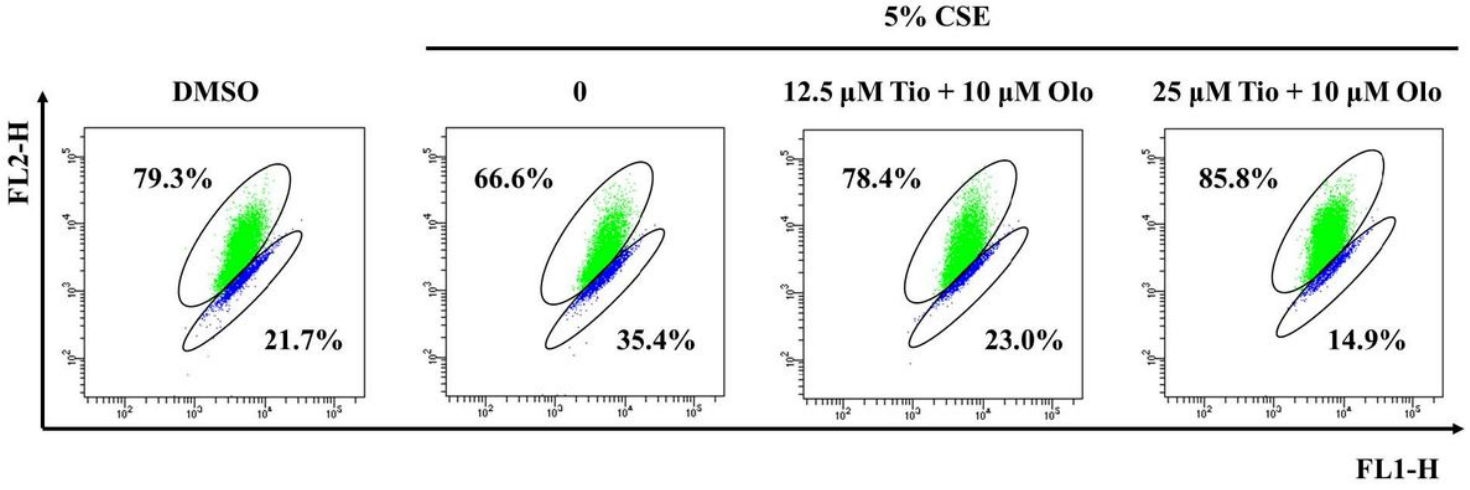

Figure 3

Effect of Tiotropium/olodaterol on CSE-mediated MMP change in BEAS-2B cells. BEAS-2B cells were pre-treatement with Tiotropium/olodaterol for 4h, followed by treatment with or without 5% CSE for 24h. Then, JC-1 staining and analyzed by flow cytometry. Red fluorescence represents cells with normal mitochondria membrane potential and green fluorescence represents those with depolarized mitochondrial membrane. Tio: tiotropium; Olo: Olodaterol

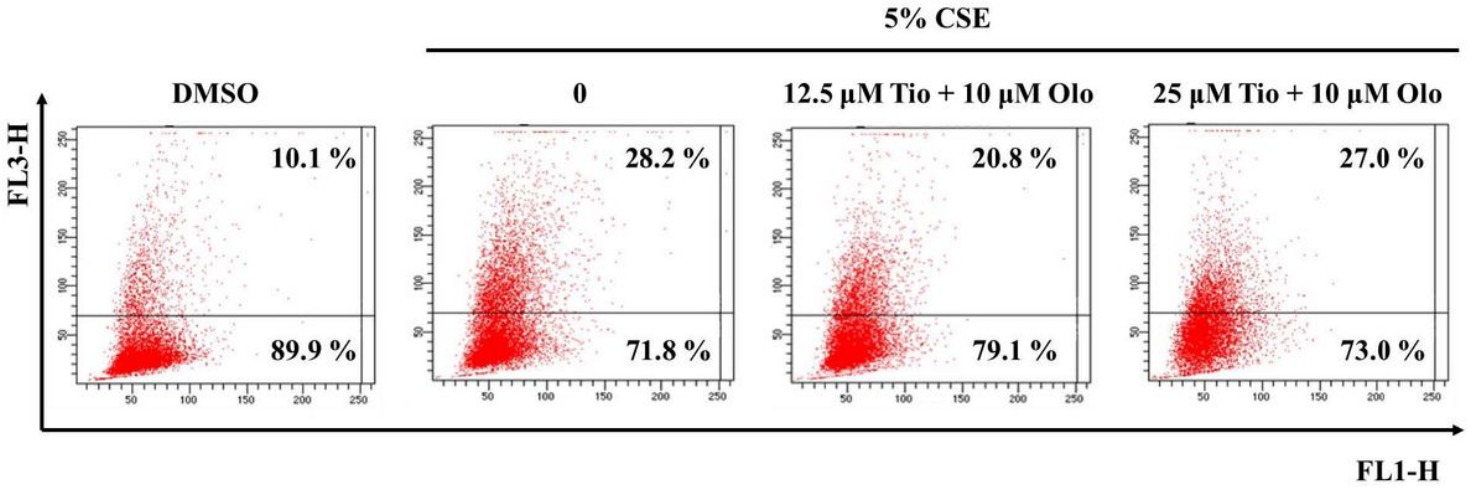

Figure 4

Effect of Tiotropium/olodaterol on CSE-mediated autophagy in BEAS-2B cells. (A) BEAS-2B cells were pre-treatement with Tiotropium/olodaterol for 4h, followed by treatment with or without 2.5% and 5% CSE for 24h. Then, AVO staining and analyzed by flow cytometry. AO accumulates and aggregates are characterized by yellow-orange fluorescence and normal cells with AO is characterized by green fluorescence.

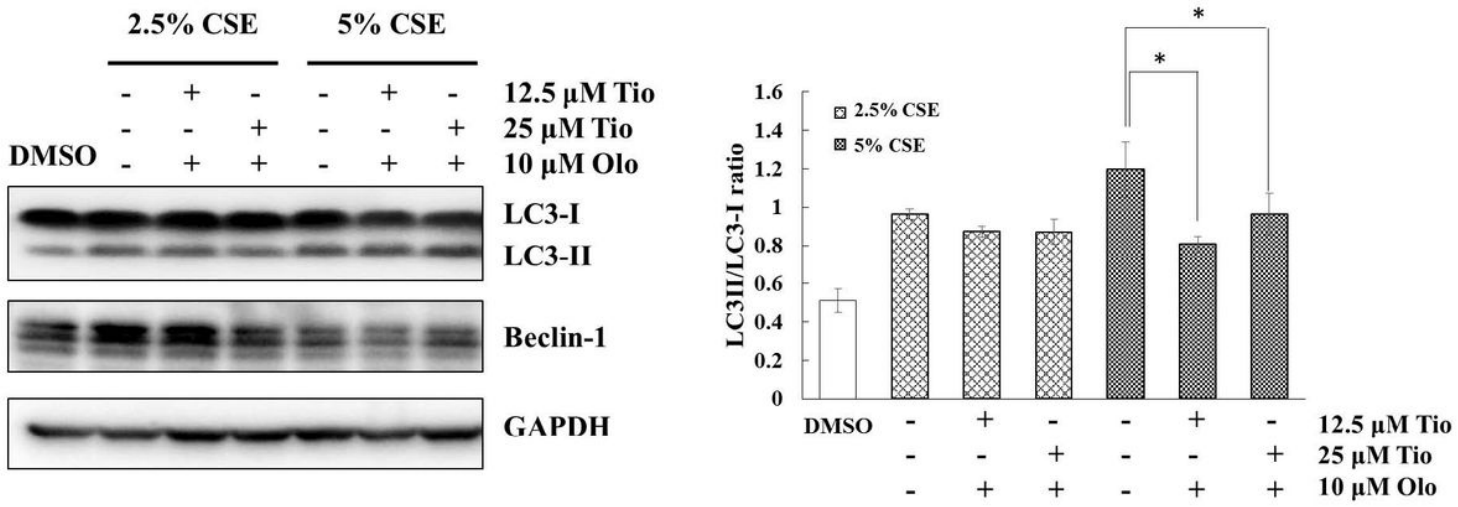

Figure 5

(B) Western blot analysis of the expression of LC3 and Beclin 1 BEAS-2B cells. Tio: tiotropium; Olo: Olodaterol

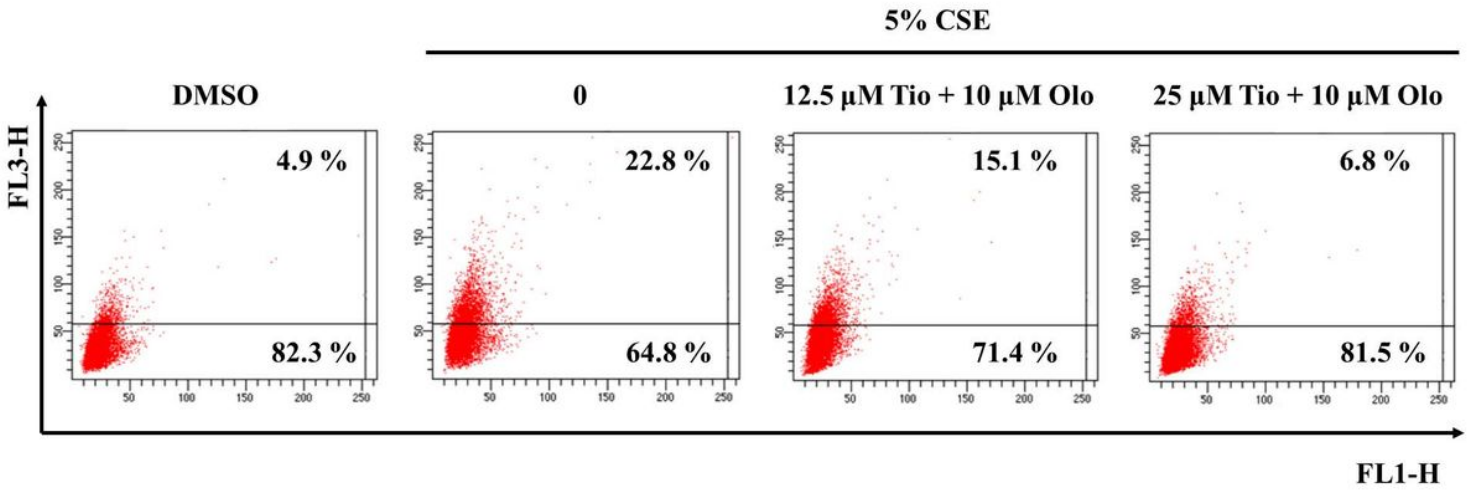

Figure 6

Effect of Tiotropium/olodaterol on CSE-mediated autophagy in BEAS-2B cells. (A) BEAS-2B cells were pre-treatement with Tiotropium/olodaterol for 4h, followed by treatment with or without 2.5% and 5% CSE for 6h. Then, AVO staining and analyzed by flow cytometry. AO accumulates and aggregates are characterized by yellow-orange fluorescence and normal cells with AO is characterized by green fluorescence.

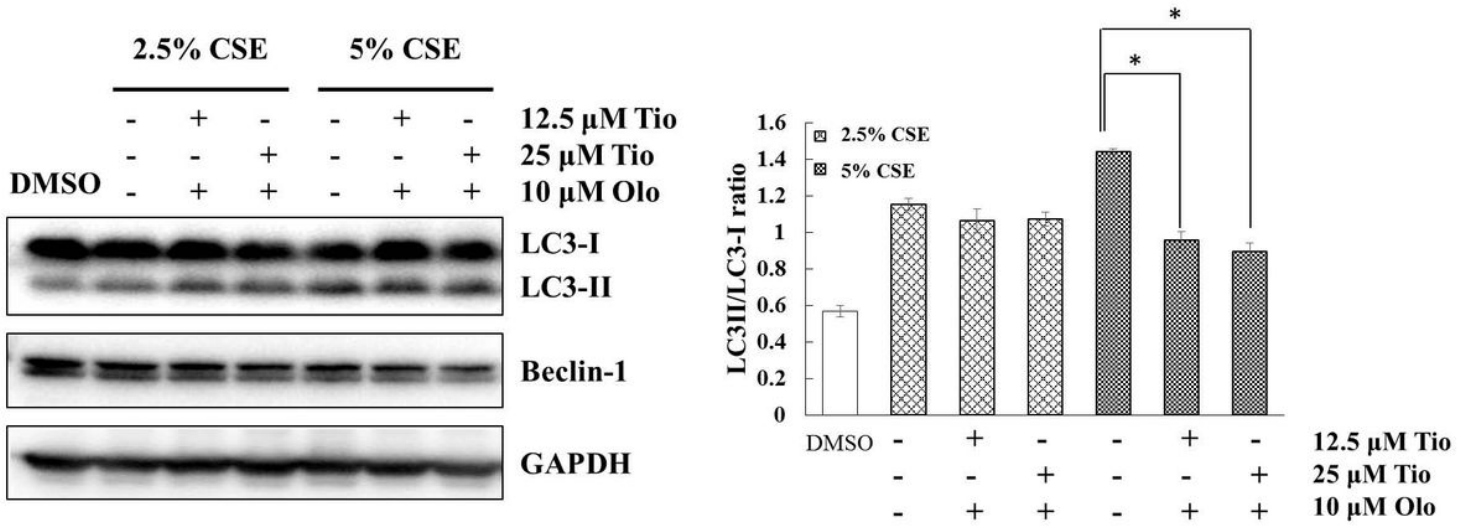

Figure 7

(B) Western blot analysis of the expression of LC3 and Beclin 1 BEAS-2B cells. Tio: tiotropium; Olo: Olodaterol

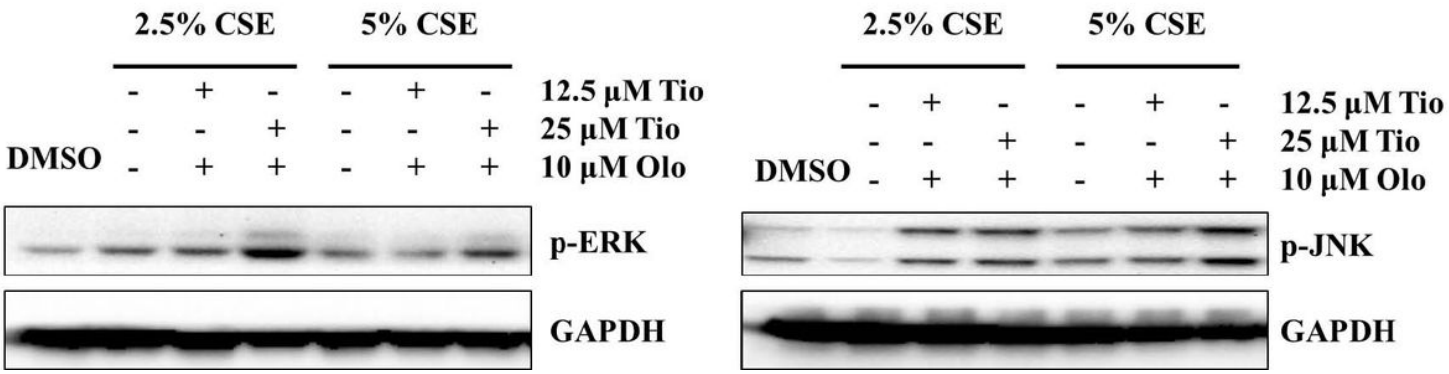

Figure 8

Effects of of Tiotropium/olodaterol on ERK and JNK activation. A) BEAS-2B cells were pre-treatement with Tiotropium/olodaterol for 4h, followed by treatment with or without 2.5% and 5% CSE for 3h. Western blot analysis of the expression of phosphor ERK in BEAS-2B. B) BEAS-2B cells were pre-treatement with Tiotropium/olodaterol for 4h, followed by treatment with or without 5% CSE for 6h. Western blot analysis of the expression of phosphor JNK in BEAS-2B. Tio: tiotropium; Olo: Olodaterol