Bronchial thermoplasty decreases airway remodelling by inhibiting autophagy via the AMPK/mTOR signaling pathway

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Bronchial thermoplasty decreases airway remodeling by inhibiting autophagy via the AMPK/mTOR signaling pathway

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Abstract

Bronchial thermoplasty (BT), an effective treatment for severe asthma, requires heat to reach the airway to reduce the mass of airway smooth muscle cells (ASMCs). Autophagy is involved in the pathological process of airway remodelling in patients with asthma. However, it remains unclear whether autophagy participates in controlling airway remodelling induced by BT. In this study, we aim to elucidate the autophagy-mediated molecular mechanisms in BT. Our study revealed that the number of autophagosomes and the level of alpha-smooth muscle actin (α-SMA) fluorescence were significantly decreased in airway biopsy tissues after BT. As the temperature increased, BT caused a decrease in cell proliferation and a concomitant increase in the apoptosis of human airway smooth muscle cells (HASMCs). Furthermore, increases in temperature significantly
downregulated cellular autophagy, autophagosome accumulation, the LC3II/LC3I ratio, and Beclin-1 expression, upregulated p62 expression, and inhibited the AMPK/mTOR pathway. Furthermore, cotreatment with AICAR (an AMPK agonist) or RAPA (an mTOR antagonist) abolished the inhibition of autophagy and attenuated the increase in the apoptosis rate of HASMCs induced by the thermal effect. Therefore, we conclude that BT decreases airway remodelling by blocking autophagy induced by the AMPK/mTOR signaling pathway in HASMCs.

Keywords: bronchial thermoplasty, autophagy, airway remodelling, apoptosis, AMPK/mTOR

Introduction

Asthma is a lung disease characterized by reversible airway obstruction, airway inflammation, and increased airway responsiveness to multiple stimuli. Severe asthma may cause shortness of breath and chest tightness and may be life-threatening. The main treatment for asthma is drug therapy; however, such treatment can cause side effects and lead to drug tolerance [1-3]. Therefore, identifying nonpharmacological treatments for asthma is important. Bronchial thermoplasty (BT) is a nondrug procedure that can be used to treat patients with severe persistent asthma, and these patients are unable to effectively control their asthma by the use of corticosteroids and long-acting beta-agonists. BT can reduce acute attacks, exacerbation rates and hospitalization rates and thus improve the quality of life of asthma patients [4-6].

A previous report revealed that BT could reduce airway hyperresponsiveness via the effects of radiofrequency energy [7, 8]. It has been reported that thermoplasty may induce epithelial cells to regenerate [9]. Moreover, BT can inhibit fibroblast remodelling by changing the function of epithelial cells [10]. BT is currently believed to exert therapeutic effects by changing the structure or function of airway smooth muscle (ASM) tissue [11], airway nerves [12, 13], airway glands [14], and airway vasculature and reducing airway inflammation [15, 16]. BT has also been reported to change the function of ASM tissue by diminishing its mass [17]. While silencing miR-10b-5p was shown to reduce the hypertrophy of human aortic smooth muscle cells (HASMCs) by downregulating FLT1 and upregulating PI3K/Akt [18], the detailed signaling mechanism of BT in asthma has rarely been studied.

Recent studies have shown that autophagy is related to asthma pathogenesis, which involves both innate and adaptive immune responses [19]. In asthma, an increase in autophagy enhances
the type 2 immune response and eosinophilic inflammation, while autophagy is decreased in
neutrophilic asthma [20]. It has been reported that miR-192-5p may attenuate airway remodeling
and autophagy in asthma via the MMP-16 and ATG7 pathways [21]. However, whether
autophagy is involved in airway remodeling mediated by BT is unknown.

Here, we constructed a BT cell model and used it to explore the effect of temperature on the
proliferation of HASMCs. Our data showed that autophagy may be involved in the mechanism by
which BT diminishes the ASM mass. Our study also provides experimental data and hypotheses
concerning how BT improves the function of HASMCs.

Materials and Methods

Study cohort
All enrolled patients were diagnosed according to the Global Initiative for Asthma guidelines. The
patients had received high doses of inhaled corticosteroids (ICSs) during the preceding year and
were currently using long-acting β-agonists (LABAs). None of the patients had been previously
treated with omalizumab or endotracheal intubation plus mechanical ventilation. BT was
performed according to a standardized protocol. The following steps were used: BT was
performed using an Alair bronchial thermoforming system (Asthmatx, Sunnyvale, USA) and a
BF260 bronchoscope (Olympus, Tokyo, Japan). Under direct vision, the probe was deployed in
the airways one by one from the small airway (diameter ≥ 3 mm) to the leaf bronchial opening in
order from far to near, continuously without repeating. The first BT procedure consisted of
treatment of the right lower lobe bronchus and right main bronchus; the second session was
delivered to the left lower lobe bronchus and left main bronchus; and the third BT session was
delivered to bilateral upper lobes. All 3 BT sessions were carried out by the same experienced
respiratory interventionist. The interval between procedures was more than 3 weeks. Prednisone
(30 mg OD) was administered orally 3 days before BT, on the day of the procedure, and 1 day
after the procedure. Table 1 shows the clinical characteristics of the patients. All patients provided
written informed consent for study participation, and the study protocol was approved by the local
Institutional Ethical Committee of the University of Chinese Academy of Sciences Shenzhen
Hospital (No. LL-KT-2020140).
Cell isolation and characterization

Airway biopsy samples were collected from patients before BT (BBT) and after BT (ABT) surgery. Primary airway smooth muscle cells were isolated from the biopsy samples and cultured in DMEM (containing 20% FBS) at 37°C. The cells were subsequently cultured in DMEM (containing 10% FBS) by serial subcultivation at a density between 1:2 and 1:4. The cultured cells were identified by staining with specific α-actin (α-SMA) via an immunofluorescence assay. Cultures in which > 90% of the cells exhibited α-SMA expression were used for subsequent experiments.

Cell proliferation

A CCK-8 kit (CK04; Dojindo, Kyushu, Japan) was used to measure cell proliferation. HASMCs were seeded into 96-well plates at a density of 10,000 cells per well and cultured for 1, 3, 5, and 7 days, respectively. Next, 10 μL of CCK-8 solution was added, and the cells were incubated for 4 hours to detect cell proliferation at specific time points. The absorbance of each culture well was measured at 450 nm with a Synergy LX microplate reader (BioTek, Vermont, USA).

Experimental thermoplasty

Before BT, HASMCs were seeded into culture plates at a density of 10,000 cells/cm² and incubated overnight at 37°C. The cells were then exposed to different temperatures (37°C, 45°C, 55°C, 60°C, 65°C, 70°C) for 10 s and subsequently cultured at 37°C for the designated time periods.

Observations of autophagy vesicles and intracellular autophagosomes

Cell suspensions were prepared from fresh tissue samples and then centrifuged. Next, fixation solution (G1102; Servicebio, Wuhan, China) was added to each sample, and the cells were fixed at 4°C for 2–4 h. Afterwards, the cells were treated with 1% osmic acid (18456; Ted Pella, California, USA) in 0.1 M phosphate buffer (pH 7.4) at room temperature for 2 h. Following dehydration, the tissue samples or cells were permeated overnight with acetone and 812 embedding agent (90529-77-4; SPI, West Chester, USA). After polymerization at 60°C for 48 h, 60–80 nm ultrathin slices were prepared and stained. The slices were then examined with an
HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

**Treatment with an agonist or inhibitor**

HASMCs were exposed to a temperature of 55 or 70°C for 10 s and subsequently treated for 4 h with either 1 mM AICAR (HY-13417; MedChemExpress, Monmouth Junction, USA) or 200 nM rapamycin (RAPA) (HY-10219; MedChemExpress). The treated cells were then cultured for 3 days.

**Apoptosis detection by flow cytometry (FCM)**

HASMCs isolated from biopsy tissues before BT were seeded into culture plates at a density of 10,000 cells/cm² and incubated overnight at 37°C. After 3 days of incubation, the cells were digested to produce single-cell suspensions, and 5 µL of Annexin V-FITC and 10 µL of PI (C1062L; Beyotime, Shanghai, China) were added to stain the cells. After incubating in the dark for 15 min, the number of apoptotic cells was determined by using a CytoFLEX S flow cytometer (Beckman Coulter, California, USA). FlowJo_v10.6.2 software was used to analyse the flow cytometric data.

**Immunofluorescence assay**

HASMCs or biopsy tissues were fixed with 4% paraformaldehyde and permeabilized with Triton X-100 (T8200; Solarbio, Beijing, China). Next, the cells or tissues were incubated overnight at 4°C with primary antibodies diluted with Immunol Staining Primary Antibody Dilution Buffer (P0103; Beyotime). After washing with PBS, the cells and tissues were stained with a fluorescently labelled secondary antibody for 1 h at room temperature. Finally, DAPI staining solution (ab2285549; Abcam, Cambridge, UK) was used to stain the nuclei. The fluorescence intensity was detected under an FL-CKX3 fluorescence microscope (Olympus) or an FV3000 confocal laser scanning microscope (Olympus). The antibodies used were as follows: rabbit anti-LC3B (ab232940; Abcam), mouse anti-α-SMA (ab7817; Abcam), rabbit anti-mouse IgG H&L (Alexa Fluor®488) (ab150125; Abcam), goat anti-rabbit IgG H&L (Alexa Fluor®488) (ab150077; Abcam), and goat anti-rabbit IgG H&L (Alexa Fluor®555) (ab150078; Abcam).

**Western blotting analysis**
Lysis buffer (P0013; Beyotime) was used to extract total protein from HASMCs after different treatments. The protein concentration in each extract was determined using a BCA protein quantification kit (23227; Thermo Fisher, Waltham, USA). A sample of protein from each extract was separated by 10% SDS-PAGE, and the protein bands were subsequently transferred onto PVDF membranes (ISEQ00010; Millipore, Massachusetts, USA), which were subsequently incubated with primary antibodies against Beclin-1 (ab207612; Abcam), LC3B (ab48394; Abcam), p62 (ab109012; Abcam), p21 (ab109520; Abcam), cleaved caspase-3 (ab32042; Abcam), p-AMPK (ab131357; Abcam), AMPK (ab3759; Abcam), p-mTOR (ab109268; Abcam), mTOR (ab32028; Abcam), and GAPDH (ab9485; Abcam). Next, the membranes were incubated with a goat anti-rabbit IgG H&L (HRP) secondary antibody (ab6721; Abcam), and immunoreactivity was visualized and quantified using a Tanon 5200 Automatic Chemiluminescence Image Analysis System (Tanon, Shanghai, China).

Statistical analysis

All the experiments were conducted 3 times, and the results are expressed as the mean ± SD. Student’s t-test or ANOVA was used to compare differences between groups. The significance of differences between groups is shown as $P<0.05$.

Results

Characterization of HASMCs

HASMCs were cultured using the tissue block culture adhesion method. As shown in Figure 1A, some cells migrated from around the tissue on day 2 of culture, and the cells were in a state of fusion growth on the fifth day of culture. In the BBT group, cells cultured for the first time exhibited poor growth, possibly because the serum concentration was < 20%. To determine whether the cultured cells were SMCs, the cells were stained with a-actin, which is specific for a-SMA, during an immunofluorescence assay. As shown in Figure 1B, > 90% of the cells expressed a-SMA, indicating that the ASMCs had been successfully cultured.

Effect of temperature on HASMC proliferation and apoptosis

The CCK-8 assay was used to detect the proliferation of HASMCs. Our data showed that, compared with that in the BBT group, the proliferation of HASMCs in the ABT group was
significantly decreased on day 3 of culture (Figure 2A). Additionally, the effect of temperature on HASMC proliferation was also determined by the CCK-8 assay. HASMCs from the BBT group were exposed to different temperatures for 10 s and then cultured at 37°C for 7 days. The results showed that cell proliferation significantly decreased after exposure to temperatures of 65 or 70°C, while exposure to temperatures of 45, 55, or 60°C had little effect on cell proliferation (Figure 2B). Therefore, either 65 or 70°C was the optimum temperature for constructing a BT cell model for use in further studies.

In addition, HASMCs were collected for apoptosis detection via an FCM assay. In Figure 2C, the lower left quadrant shows living cells, the upper left quadrant shows dead cells, the lower right quadrant shows early-stage apoptotic cells, and the upper right quadrant shows late-stage apoptotic cells. The percentage of apoptotic cells was analysed by calculating the total percentage of apoptotic cells in the lower right and upper right quadrants. Our data showed that the HASMC apoptosis rate gradually increased in conjunction with increasing temperature (Figure 2D). Increased levels of p21 and cleaved caspase-3 protein expression can promote cell apoptosis [22]. Our results showed that the intracellular levels of p21 and cleaved caspase-3 increased in conjunction with increasing temperature, suggesting that an increase in temperature could increase the apoptosis rate of HASMCs (Figure 2E–G). These data indicated that the apoptosis rate of HASMSCs was increased with a rise of temperature.

Effect of BT on autophagy in patients with asthma

Clinically, we performed immunofluorescence and TEM analyses to determine the effect of BT on autophagy in patients with asthma. Immunofluorescence staining revealed that the LC3B and α-SMA fluorescence levels in airway biopsy samples from asthmatic patients after BT were significantly lower than those before BT, indicating that BT could reduce the degree of autophagy and fibrosis in airway tissue (Figure 3A). Moreover, the TEM results showed that BT reduced the number of autophagic vesicles, which are indicated by white arrows (Figure 3B and Supplementary Figure S1). These data indicated that BT could decrease the autophagic activity in patients with asthma.

Effect of temperature on autophagy and the expression of AMPK/mTOR pathway-related proteins
LC3B is widely used as a marker of autophagy, and its expression level directly reflects autophagy [23, 24]. To evaluate the effect of temperature on autophagy, HASMCs were incubated with an LC3B antibody and observed under a fluorescence microscope. An increase in temperature decreased the fluorescence intensity of LC3B, suggesting that an increase in temperature could reduce the level of autophagy in HASMCs (Figure 4A and Supplementary Figure S2A). Furthermore, as shown in Figure 4B and Supplementary Figure S2B, the number of intracellular autophagosomes decreased significantly with increasing temperature, suggesting that an increase in temperature reduced the level of autophagy in airway smooth muscle cells.

WB was performed to evaluate the effects of temperature on the expression of autophagy-related proteins (i.e., Beclin-1, LC3I, LC3II, and p62) and AMPK/mTOR pathway-related proteins in HASMCs. These results showed that as the temperature increased, the protein expression levels of Beclin-1 and LC3II in HASMCs gradually decreased, while the protein expression levels of LC3I and p62 gradually increased (Figure 4C–F). These results suggested that increasing temperature could inhibit autophagy in HASMCs. Moreover, AMPK is an important life-regulating pathway that can effectively control autophagy and inhibit mTOR [25, 26]. Our data showed that an increase in temperature significantly reduced the phosphorylation level of AMPK, while the phosphorylation level of p-mTOR dramatically increased (Figure 4C,G,F). These results indicated that increasing temperature could inhibit AMPK and promote the mTOR signaling pathway.

**Temperature affects autophagy by regulating the AMPK/mTOR signaling pathway**

The results above showed the effects of temperature on apoptosis, autophagy, and the AMPK/mTOR signaling pathway. However, whether increasing temperature mediates autophagy and apoptosis via the intracellular AMPK/mTOR signaling pathway has not been determined. Therefore, the effects of RAPA, an mTOR inhibitor, and AICAR, an AMPK agonist, on temperature-induced autophagy were examined. Our results showed that AICAR could increase the phosphorylation level of AMPK (Figure 5A,B), RAPA could reduce the phosphorylation level of mTOR (Figure 5C,D), and an increase in temperature could significantly downregulate p-AMPK/AMPK expression (Figure 5B). We also observed that the fluorescence intensity of LC3B was enhanced in both the RAPA and AICAR treatment groups (Figure 5E and Supplementary
Figure 3A). At 70°C, AICAR further increased the number of intracellular autophagosomes (red arrow), suggesting that a temperature increase affects autophagy by regulating the AMPK signaling pathway (Figure 5F and Supplementary Figure 3B). In addition, the expression levels of Beclin-1 and LC3II/LC3I were increased, while the expression level of p62 was decreased in both the RAPA and AICAR treatment groups (Figure 5G–J). We also found that an increase in temperature significantly reduced the LC3II/LC3I ratio and the level of Beclin-1 expression but markedly upregulated p62 expression (Figure 5G–J). These results indicated that both RAPA and AICAR could alleviate the effect of temperature on autophagy. Therefore, we hypothesized that an increase in temperature regulated autophagy via the cellular AMPK/mTOR signaling pathway.

Temperature affects the apoptosis of HASMCs by regulating AMPK/mTOR-mediated autophagy

FCM was used to analyse how temperature affects apoptosis via AMPK/mTOR-mediated autophagy. FCM revealed that both RAPA and AICAR could significantly reverse the damage to HASMCs caused by increasing temperature and thus reduce the percentage of apoptotic cells (Figure 6A,B). In addition, the levels of p21 and cleaved caspase-3 were decreased in both the RAPA and AICAR treatment groups (Figure 6C–E). Moreover, an increase in temperature markedly upregulated the levels of p21 and cleaved caspase-3 (Figure 6C-E). Therefore, we concluded that an increase in temperature promotes apoptosis by regulating AMPK/mTOR signaling pathway-mediated autophagy.

Discussion

Airway remodelling is one of the pathological features of severe asthma and is characterized by an increase in ASM mass, extracellular matrix (ECM) deposition, angiogenesis, and mucous gland hypertrophy. It is crucial that the masses of ASM and the ECM are increased [27]. BT is a nonpharmaceutical treatment for severe asthma and exerts its effect by imposing heat energy into the airway to improve asthma control [28]. ASM, a key factor in airway remodelling, is regarded as the main target of BT in the treatment of asthma. BT helps to diminish the ASM mass and regenerate epithelial cells [29]. A previous study showed that the numbers of airway smooth muscle cells and epithelial cells were significantly reduced along with airway smooth muscle mass...
after treatment with BT [17]. However, how BT diminishes the ASM mass during treatment has rarely been investigated. In this study, we investigated the ASM-related signaling mechanism of BT at both the clinical and cellular levels.

First, we confirmed that, with increasing temperature, HASMC proliferation decreased and HASMC apoptosis increased. In addition, the levels of p21 and cleaved caspase-3 also significantly increased with increasing temperature. These results not only were consistent with the temperature of the BT during operation but were also consistent with the results of previous studies. Therefore, modulating ASM cell apoptosis may be one of the mechanisms by which BT improves asthma. However, there have been few reports concerning the specific mechanism by which BT induces apoptosis in ASM cells. Apoptosis is a common form of programmed cell death and is related to many factors. As one of the important factors, autophagy is a physiological process during which eukaryotic cells self-renew their intracellular proteins and organelles.

Autophagy has both prosurvival and prodeath effects. On the one hand, autophagy can promote the survival of cells by removing damaged organelles to maintain cell homeostasis; on the other hand, excessive levels of autophagy also promote cell death via apoptotic mechanisms. S. [30] An increasing number of studies have shown that autophagy plays an important role in the pathogenesis of asthma [31-35]. In asthma, with significant activation of autophagy, autophagy becomes involved in airway remodelling; however, inhibition of autophagy can significantly improve this pathological process [36]. In our study, we first observed that autophagy levels in airway tissue and HASMCs isolated in vitro were significantly reduced after BT, revealing that thermal treatment could inhibit autophagy at both the clinical and cellular levels. In addition, we observed that the apoptosis of HASMS induced by BT was increased. Therefore, the dysregulated balance between autophagy and apoptosis may be involved in the intervention of BT.

Previous studies have shown that temperature can affect cellular autophagy in a temperature-dependent manner. An increase in temperature within a certain range can activate cellular protective autophagy [37-39]. However, a temperature increase can also produce certain ramifications that depend on the amount of increase. An early study described the effect of a physiologically relevant increase in temperature on autophagy induction in isolated rat hepatocytes. In that study, the highest autophagy levels were detected at 37°C, which is the normal body temperature, whereas an increase in temperature to 40°C reduced autophagic
sequestration. Another study showed that heat exposure could trigger autophagy in human alveolar basal epithelial cells. The above findings indicate that heat exerts complex effects on autophagy.

In summary, the combined results from previous studies and our current study indicate that temperature influences autophagy. Additionally, there may be a threshold at which temperature affects autophagy. A temperature threshold < 43°C mainly activates protective autophagy in cells [37]. Our results showed that autophagy in smooth muscle cells was inhibited when the temperature reached the temperature range of 70°C. This finding explains why BT must reach an average temperature of 70°C to effectively treat asthma. On the other hand, thermal effects may regulate autophagy via indirect signaling pathways. Heat stress denatures proteins and alters mRNA translation [38]. Increasing evidence indicates that BT can upregulate the expression of heat shock proteins in airway epithelial tissues [10, 40]. Studies using heat treatment have shown a close relationship between heat shock proteins and autophagy [38]. Overexpression of HSP70 was found to suppress the autophagic response during starvation-induced autophagy in cultured A549 cells [41]. Peter et al. [42] showed that 15 minutes of thermal treatment could not cause autophagy. Therefore, it is crucial to investigate the signaling mechanism by which BT affects autophagy.

The AMPK-mTOR pathway is an important pathway that regulates autophagy. Previous studies revealed that AMPK effectively controls autophagy and inhibits mTOR [26, 43]. Wen et al. [39] reported that heat treatment of lung cancer cells in combination with chloroquine could regulate the AMPK-mTOR signaling pathway, inhibit cell protective autophagy, and promote cell apoptosis. Fang et al. [9] investigated the function of heat shock proteins in BT and speculated that HSP70 regulates the AKT-mTOR signaling pathway to produce an epithelial cell rescue effect. In our study, we found that increasing heat exposure inhibited autophagy by suppressing AMPK and promoting the mTOR signaling pathway. In addition, it was further verified that an elevated heat treatment promoted the apoptosis of ASM cells by regulating AMPK/mTOR signaling pathway-mediated autophagy.

It has been reported that the inhibition of autophagy in autophagy-related (ATG) 5 and ATG7 conditional knockout mouse models leads to degenerative changes in muscle tissue, a reduction in myofiber size, the accumulation of protein aggregates and abnormal mitochondria, and a reduction
in muscle force that leads to severe weakness [44, 45]. Similarly, for ASM, our results suggest that BT can inhibit autophagy in ASM cells by regulating the AMPK-mTOR pathway, inducing apoptosis, and decreasing the number of ASM cells. Therefore, we speculate that BT might reduce the contractile function of smooth cells through this effect, which may be one of the mechanisms by which BT is effective in treating asthma.

However, our study has several limitations: (1) the effect of BT on immune cells needs to be further studied; (2) transcriptional and proteomic studies of clinical tissues and cells before and after BT need to be conducted; and (3) metabolomic studies of bronchoalveolar lavage fluid (BALF) before and after BT need to be performed.

In conclusion, we investigated the mechanisms by which BT improves ASM at both the clinical and cellular levels and found that the thermal effect of BT enhanced the apoptosis of ASM cells by regulating AMPK/mTOR signaling pathway-mediated autophagy (Figure 7). Our study reveals the specific ASM-related signaling mechanism by which BT enhances HASMCs.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

**References**


2. Gans, M.D. and T. Gavrilova, *Understanding the immunology of asthma: Pathophysiology, biomarkers, and treatments for asthma endotypes*. Paediatr Respir


apoptosis and transcriptional regulation after DNA damage. DNA Repair (Amst), 2016.


**Figure legends**

**Figure 1. Culture and characterization of HASMCs in the ABT and BBT groups**  (A) HASMCs were cultured 2 times by the tissue block culture adhesion method. The cells were observed on the second and fifth culture days under a microscope. (B) Identification of HASMCs stained with an a-SMA antibody via an immunofluorescence assay. BBT: before BT; ABT: after BT.

**Figure 2. Effect of temperature on the proliferation and apoptosis of HASMCs**  (A) The proliferation of HASMCs cultured for 1 day, 3 days, 5 days, or 7 days in the BBT and ABT groups was detected by the CCK-8 assay. ***P<0.001 represents the BBT group vs. the ABT group. (B) Proliferation of HASMCs exposed to different temperatures (37, 45, 55, 60, 65, and 70°C) for 10 s. Absorbance was measured at 450 nm. ***P<0.001 vs 37°C. (C) HASMCs in the BBT group were subjected to heat intervention (37, 45, 55, 60, 65, and 70°C) for 10 s and then cultured at 37°C for 3 days; after which, apoptosis was analysed via FCM. (D) Statistical histogram of the apoptosis rates in the different heat intervention groups. *P < 0.05, ***P < 0.001 vs 37°C. (E) The effect of temperature on p12 and cleaved caspase-3 expression in HASMCs was evaluated by western blotting. (D) Statistical histogram of p12 and cleaved caspase-3 expression in the different heat intervention groups. *P < 0.05, **P < 0.01, and ***P < 0.001 vs 37°C.

**Figure 3. Effect of BT on autophagy in patients with asthma.**  (A) Immunofluorescence analysis of airway biopsy samples from patients with asthma before and after BT. (B) TEM analysis of airway biopsy samples from patients with asthma before and after BT. The white arrows indicate autophagy vesicles.
Figure 4. Effects of different temperatures (37°C, 45°C, 55°C, 60°C, 65°C, 70°C) on autophagy and AMPK/mTOR pathway-related proteins in primary airway smooth muscle cells (A) The effect of temperature on LC3B expression in HASMCs was evaluated by immunofluorescence. Nuclei were stained with DAPI. (B) TEM analysis of intracellular autophagosomes, which are shown by red arrows. (C) The effect of temperature on Beclin-1, LC3I, LC3II, and p62 expression in HASMCs was evaluated by western blotting. (C) The effect of temperature on the expression of AMPK/mTOR pathway-related proteins (p-mTOR, mTOR, p-AMPK, and AMPK) in HASMCs was evaluated by western blotting. GAPDH served as a reference protein. p-: phosphorylated. A statistical histogram of Beclin-1 (D), LC3II/LC3I (E), p62 (F), p-AMPK/AMPK (G), and p-mTOR/mTOR (H) expression was generated at different heat treatment temperatures. *P < 0.05, **P < 0.01 and ***P < 0.001 represent vs. 37°C.

Figure 5. The effect of temperature (55°C and 70°C) on autophagy is also mediated by the AMPK/mTOR signaling pathway (A) The levels of p-AMPK and AMPK were detected via western blotting. (B) Statistical histogram of p-AMPK/AMPK expression. (C) The levels of p-mTOR and mTOR were detected via western blotting. (D) Statistical histogram of p-mTOR/mTOR expression. (E) Immunofluorescence assays were performed in which HASMCs were exposed to a temperature of 55°C or 70°C for 10 s and then treated with AICAR or RAPA. HASMCs were subsequently stained with LC3B, and cell nuclei were stained with DAPI. (F) Intracellular autophagosomes (red arrows) were observed via TEM. (G) Beclin-1, LC3I, LC3II, and p62 expression was detected via western blotting. HASMCs were exposed to a temperature of 55°C or 70°C for 10 s and then treated with AICAR or RAPA. A statistical histogram of Beclin-1 (H), LC3II/LC3I (I), and p62 (J) expression after different heat interventions is shown. *P < 0.05, **P < 0.01 and ***P < 0.001 represent the control group. #P < 0.05, ##P < 0.01 and ###P < 0.001 represent vs. 55°C.

Figure 6. Temperature (55°C and 70°C) regulated apoptosis by affecting AMPK/mTOR signaling pathway-mediated autophagy (A) The apoptosis of HASMCs treated with RAPA or AICAR was analysed via FCM. (B) Statistical histogram of the apoptosis rate data. (C) The levels
of p21 and cleaved caspase-3 were detected via western blotting. HASMCs were exposed to a
temperature of 55°C or 70°C for 10 s and then treated with RAPA or AICAR. A statistical
histogram of p21 expression (D) and cleaved caspase-3 expression (E) is shown. * P < 0.05, ** P
< 0.01 and *** P < 0.001 vs the control group. # P < 0.05, ## P < 0.01 and ### P < 0.001 represent
vs. 55°C.

**Figure 7.** BT enhanced the apoptosis of ASM cells by regulating AMPK/mTOR signaling
pathway-mediated autophagy
176x77mm (300 x 300 DPI)
73x60mm (300 x 300 DPI)
163x135mm (300 x 300 DPI)
Bronchial thermoplasty

Increased temperature

AMPK

mTOR

Autophagosome

Apoptosis

Airway smooth muscle cells

115x91mm (300 x 300 DPI)
Table 1. Clinical characteristics of the patient cohorts

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<td>3.07 ± 1.36</td>
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<tr>
<td>AQLQ</td>
<td>3.67 ± 0.91</td>
</tr>
<tr>
<td>FENO (ppb)</td>
<td>71.13 ± 90.75</td>
</tr>
<tr>
<td>Total serum IgE (IU/mL)</td>
<td>311.78 ± 275.76</td>
</tr>
<tr>
<td>Peripheral eosinophil count (×10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>0.72 ± 1.55</td>
</tr>
<tr>
<td>ICS/LABA (n)</td>
<td>15</td>
</tr>
<tr>
<td>∆FVC*</td>
<td>0.11 ± 0.63</td>
</tr>
<tr>
<td>∆FEV&lt;sub&gt;1&lt;/sub&gt;*</td>
<td>0.49 ± 0.62</td>
</tr>
<tr>
<td>∆Post-BD FEV&lt;sub&gt;1&lt;/sub&gt;% pred*</td>
<td>8.73 ± 18.60</td>
</tr>
<tr>
<td>∆ACT*</td>
<td>7.93 ± 5.51</td>
</tr>
<tr>
<td>∆ACQ*</td>
<td>−1.96 ± 1.52</td>
</tr>
<tr>
<td>∆AQLQ*</td>
<td>1.77 ± 0.96</td>
</tr>
</tbody>
</table>

Data were expressed as the mean ± SD or number, percentage, unless otherwise stated. BMI: body mass index; FVC: forced vital capacity; FEV<sub>1</sub>: forced expiratory volume in 1 s; BD: bronchodilator; ACT: asthma control test; AQLQ: asthma quality of life questionnaire; FENO: exhaled nitric oxide fraction; ICS: inhaled corticosteroid; LABA: long-acting β-agonist. *Δ means
Change from baseline over 1 month after BT.
Supplementary Figure S1. Effect of BT on autophagy in patients with asthma. The number of autophagosomes per field was quantitatively analysed via transmission electron microscope. $** P < 0.01$. 
Supplementary Figure S2. Effects of different temperatures on autophagy in primary airway smooth muscle cells  (A) The relative cytoplasmic intensity of LC3B was statistically analysed. (B) The number of autophagosomes per field was quantitatively analysed via TEM. *P < 0.05, **P < 0.01 and ***P < 0.001 vs 37°C.
Supplementary Figure S3. Effects of temperature (55°C and 70°C) on autophagy via the AMPK/mTOR signaling pathway  

(A) The relative cytoplasmic intensity of LC3B was statistically analysed. (B) The number of autophagosomes per field was quantitatively analysed via TEM. *P < 0.05 and *** P < 0.001 represent the control group. # P < 0.05 and ## P < 0.01 vs 55°C.
**Highlights**

Bronchial thermoplasty (BT), as an effective treatment for severe asthma, imposes heat energy into the airway to reduce the mass of airway smooth muscle cells (ASMCs). Autophagy is involved in the pathological process of airway remodeling in asthma. Here, our study reveals that enhances the apoptosis of ASMCs by regulating AMPK/mTOR signaling pathway-mediated autophagy. 

1. Temperature increasing enhances the apoptosis rates of airway smooth muscle cells.
2. Temperature increasing inhibits the AMPK/mTOR signaling pathway-mediated autophagy.
3. BT decreases airway remodeling by blocking AMPK/mTOR signaling pathway induced autophagy.