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The role of autophagy in the overexpression of *MUC5AC* in patients with chronic rhinosinusitis☆

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ABSTRACT

Background: Autophagy is a lysosomal degradation pathway that protects the body and is essential for cell survival and differentiation. Mucins (MUCs) are important components of secreted mucus, mucin (MUC)5 AC is the major MUC secreted in the normal airway.

Objective: Investigated the role of autophagy in pathogenic mucin (MUC)5 AC production during chronic rhinosinusitis (CRS).

Methods: The expression of human neutrophil elastase (HNE) and the autophagic proteins microtubule-associated protein 1 light chain (LC)3B-II, c-Jun N-terminal kinase (JNK), c-Jun, and MUC5AC were analyzed in the sinonasal mucosa and human nasal epithelial cells (HNECs) using immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and quantitative real-time polymerase chain reaction (qRT-PCR). Autophagic vacuoles were studied using transmission electron microscopy (TEM). Primary HNECs were treated with HNE, bafilomycin A1, and SP600125. In some experiments, cultured primary HNECs were transfected with small interfering RNAs (siRNAs) to target Beclin-1 (BECN1; BECN1-siRNA), autophagy-related gene 5 (Atg5; Atg5-siRNA), and c-Jun (c-Jun-siRNA). Cultured cells were analyzed using western blotting, qRT-PCR, and ELISA.

Results: In CRS patients, both with and without nasal polyps, the expression levels of HNE, LC3B, JNK, c-Jun, and MUC5AC were upregulated. Bafilomycin A1 upregulated LC3B-II expression and inhibited MUC secretion in HNE-treated normal primary HNECs. Autophagosomes were observed in HNE-treated primary HNECs using TEM. HNE-induced secretion of MUC5AC was suppressed in normal primary HNECs by BECN1-siRNA, Atg5-siRNA, c-Jun-siRNA, and SP600125.

Conclusions: In HNE-induced CRS, autophagy increases the secretion of MUC5AC by promoting the phosphorylation of JNK and c-Jun.

1. Introduction

Chronic rhinosinusitis (CRS) is a multifactorial upper airway disorder involving ongoing inflammatory reactions of the sinonasal mucosa. Excessive amounts of nasal and sinus mucus are secreted, which has a significant impact on the quality of life of patients. The condition affects 4–10% of the global population. CRS is generally classified into two subtypes: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP) [1]. Despite advances in medical and surgical therapy, CRS often recurs after treatment, particularly CRSwNP [2]. This is partly because CRS pathogenic mechanisms are not well understood and remain controversial [2,3]. Mucins (MUCs) are important components of secreted mucus, which is synthesized by epithelial goblet cells and submucosal mucous glands. Goblet cell hyperplasia and increased MUC expression are important pathological characteristics of both CRSsNP and CRSwNP, and they account for common symptoms, such as rhinorrhea and nasal congestion [4]. Several MUCs, including MUC2, MUC4, MUC5AC, and MUC5B, are also expressed in human airways. Among these, MUC5AC is the major MUC secreted in the normal airway, and MUC5AC expression is significantly upregulated in the sinus mucosa of patients with CRS [5,6]. High-density MUC glycoproteins can alter the elasticity and viscosity of airway mucus. Our previous study found that human neutrophil elastase (HNE) was significantly increased in CRS lesions compared to normal tissues [7]. We also showed that HNE stimulation promoted the secretion of MUC5AC in an in vitro culture of primary human nasal epithelial cells (HNECs).

☆ There are no financial or other issues that might lead to conflict of interest.

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Although various studies have investigated MUC hypersecretion in CRS [6,8], the molecular mechanisms underlying mucus hypersecretion and the overexpression of MUC5AC are not completely understood.

Autophagy is a dynamic process responsible for the turnover of cellular organelles and long-lived proteins. It plays a pivotal role in cellular homeostasis and adaptation to adverse environments [9]. Autophagy is also a lysosomal degradation pathway for damaged organelles and proteins. During autophagy, the outer membrane of an autophagosome fuses with the lysosome to form an autolysosome, leading to degradation of the material within [10]. This process is essential for cell survival, differentiation, development, and homeostasis [11]. > 30 autophagy-related genes (ATGs) and gene products have been identified in yeast and higher mammals. These include Atg5, Atg12, Beclin-1 (BECN1), which is encoded by the BECN1 gene and is a mammalian ortholog of yeast ATG6, and the microtubule associated protein 1 light chain (LC)3B, which is an ortholog of yeast Atg8 [12,13]. In mammals, the conversion of free LC3B (i.e., LC3B-I) to a phosphatidylethanolamine-conjugated form (i.e., LC3B-II) is a key step in autophagosome formation. The quantity of LC3B-II and the ratio of LC3B-II to LC3B-I can be used to monitor autophagy in mammalian cells, and LC3B-II levels are widely used as a marker for autophagosomes [14]. In addition, other autophagic proteins, including Atg3, Atg5, Atg7, and BECN1, are also involved in the formation of autophagosomes [15]. Therefore, upregulated expression of LC3B-II and other autophagic proteins, together with an increase in autophagic vacuoles, usually indicates that autophagy has been induced, provided that autophagosome clearance has not been impaired [14].

Recent research has shown that autophagy plays an important role in some diseases, including airway disorders [16–19]. In chronic obstructive pulmonary disease (COPD), autophagy can promote fibroblast and lung epithelial cell death in response to cigarette smoke and contributes to the development of emphysema [17]. In asthma, autophagy gene polymorphisms can increase the susceptibility of airway epithelial cells to severe respiratory viral infections [18]. Emerging evidence also suggests that autophagy plays an important role in CRS [20–23]. One recent study showed that LC3II expression is elevated in nasal polyps, in which autophagy is involved [24]. Three other preliminary studies reported conflicting results on the role of autophagy in nasal polyp-derived fibroblasts. Therefore, further studies will be necessary to clarify the role of autophagy in CRS pathogenesis [24–26]. Research has confirmed that there is a correlation between autophagy and the expression of MUC5AC. Studies showed that ultrafine particulate matter or interleukin (IL)-13 had to be present to mediate autophagy and overexpress MUC5AC in human tracheal epithelial cells. In addition, inhibition of autophagy can reduce the airway inflammation and mucus hypersecretion caused by particulate matter or IL-13 [27,28].

This study addresses these questions using in vitro and in vivo pharmacological and genetic approaches. Our results demonstrate that HNE triggers autophagy in nasal epithelial cells, and that autophagy is essential for the subsequent HNE-induced mucus hypersecretion. In addition, we investigated the downstream signaling pathways that mediate HNE-induced autophagy and MUC expression. We also demonstrated that autophagy regulates the expression of MUC5AC via c-Jun N-terminal kinase (JNK) and activator protein-1 (AP-1).

2. Materials and methods

2.1. Subjects

A total of 30 CRS patients (15 CRSwNP and 15 CRSsNP patients) were included in this study. The CRSwNP and CRSsNP diagnoses were based on patients' clinical histories and anterior rhinoscopy/nasal endoscopy/computed tomography results in accordance with the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines [1]. Oral glucocorticoids and intranasal steroid sprays were discontinued at least 3 months and 1 month before surgery, respectively. Polyp tissue from CRSwNP patients and uncinate processes from CRSsNP patients were sampled under anesthesia. The control group included 15 patients with simple nasal septum deviations who had undergone inferior turbinate tissue and nasal septum correction procedures. Endoscopic surgery was performed on the uncinate mucosa of patients with maxillary sinus mucosal retention cysts. Subjects who had antrochoanal polyps, cystic fibrosis, fungal sinusitis, primary ciliary dyskinesia, or gastroesophageal reflux disease were excluded from the study. Control subjects were free of nasal symptoms, had no history of atopic disease, and had negative skin-prick test results for common allergens. Demographic data for the CRS patients and control subjects enrolled in this study are shown in Table 1. The study protocols were approved by the Review Board for Human Studies of the First Affiliated Hospital of Nanchang University (Nanchang, China), and written informed consent was obtained from each participant or their legally authorized representative. Each specimen was separated into three portions. One portion each was immediately frozen in liquid nitrogen and stored at −80 °C for mRNA and protein analyses. A third portion was used for histological staining.

Table 1
Clinical characteristics of the CRS patients and the normal control subjects.

	Control	CRSsNP	CRSwNP
Subject no	15	15	15
Age (year (range))	16–73	17–67	7–71
Gender	9 M/6 F	9 M/6 F	7 M/8F
Atopy	0	1	0
History of asthma	0	0	0
Aspirin intolerance	0	0	0
Antrochoanal polyp	0	0	0

CRS, chronic rhibosinusitis; CRSsNP, chronic rhibosinusitis without nasal polyps; CRSwNP, chronic rhibosinusitis with nasal polyps; M, male; F, Female.

2.2. Immunohistochemistry

Standard immunohistochemical procedures were applied to the human nasal tissues. The sections were stained using mouse monoclonal antibodies and rabbit monoclonal/polyclonal antibodies against the following proteins: HNE (1:200; Novus Biologicals, Littleton, CO, USA), LC3B (1:200; Sigma-Aldrich, St. Louis, MO, USA), JNK1 and JNK2 (1:400; Abcam, Cambridge, UK), c-Jun (1:250; Abcam), and MUC5AC (1:400; Abcam). The antibodies were detected using streptavidin-biotin-horseradish peroxidase. The immunostaining results were considered positive when brown cells appeared following treatment with 3,3′-diaminobenzidine. Primary antibodies were replaced with isotype-matched immunoglobulin G (IgG) antibodies for negative control treatments. The staining patterns in epithelial cells and submucosal gland tissue were analyzed using Image J analysis software (NIH, Bethesda, MD, USA) and the results are presented as average optical density values per unit area [22].

2.3. Quantitative real-time polymerase chain reaction

Total RNA was extracted from nasal tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized from 2 μg of total RNA using an oligo (dT) 18 primer and M-MLV reverse transcriptase (TaKaRa Bio, Inc., Shiga, Japan). We performed quantitative real-time polymerase chain reaction (qRT-PCR) using the ABI PRISM 7600 detection system (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq (TaKaRa Bio, Inc.). The primer sequences used for the HNE, BECN1, ATG5, MUC5AC and β-ACTIN genes are listed in Table 2. The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (Ct). The mRNA expression level for each gene was calculated as the difference

Table 2
Primers used for quantitative real time PCR analysis.

Species	Genes	Primer sequence (5′–3′)
Human	β-actin	Forward: 5′-attgccgacaggatgcagaa-3’ Reverse: 5′-ctgatccacatctgctgga-3′
Human	HNE	Forward:5′-actcgcgtgtcttttcctcg-3’ Reverse:5′-acgaagttgggcgcaatca-3′
Human	BECN1	Forward: 5′- tgagggatggaagggtctaa-3’ Reverse: 5′-cctgggctgtggtaagtaatg-3′
Human	ATG5	Forward: 5′- gctgtgatgaagaaagtgtgg-3’ Reverse: 5′- agggcattgtaggcttgact-3’
Human	MUC5AC	Forward: 5’-actggggagaaatatcgcatg-3’ Reverse: 5′-ctgatcaaagggcctgatagc-3’

HNE, human neutrophil elastase; BECN1, Beclin-1; ATG5, autophagy-related gene 5.

(ΔCt) between the Ct value of the target gene and the Ct value of β-ACTIN. The fold change in target gene mRNA levels was calculated as $2^{-\Delta\Delta Ct}$.

2.4. Enzyme-linked immunosorbent assay

Tissue samples were weighed and homogenized. Supernatants were collected and tissue lysates were prepared by adding 1 mL of phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Keygentec, Nanjing, China) to 100 mg of tissue [5,7]. Protein levels were measured per mL of tissue homogenate and cell culture supernatant. Protein concentrations were determined using the bicinchoninic acid assay method. The levels of secreted HNE and MUC5AC proteins in each supernatant were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) procedure developed in our laboratory using anti-HNE and anti-MUC5AC antibodies (Aviva Systems Biology, San Diego, CA, USA). A total of 10 μL of sample and 40 μL of diluent were added to 96-well plates pre-coated with the captured HNE and MUC5AC antibodies. After incubation and washing, the anti-HNE and anti-MUC5AC antibodies were added to each well. After further incubation and washing, 100 μL of horseradish peroxidase conjugated goat anti-mouse IgG was added to each well. The colorimetric reaction was developed using 3,3′,5,5′-tetramethylbenzidine peroxide solution and stopped using 1 mol/L sulfuric acid. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA), and the optical density value at 450 nm was recorded. The amount of HNE in each sample was normalized to the total protein in the tissue lysates and expressed as the optical density value per mg of total tissue protein.

2.5. Primary culture and treatment of nasal epithelial cells: HNE preparation, treatment and transfection with siRNA

Immediately after surgical removal, each specimen was washed with sterile saline to remove mucus and blood and placed in a sterile centrifuge tube with 5 mL of 10% double antibody in PBS. Specimens were immediately transferred to the laboratory on ice and washed three times with 10% double antibody in PBS. It was unnecessary to cut or separate specimens to avoid mucosal damage. The submucosal tissue had been degraded enzymatically, resulting in impurities. Each specimen was placed in a 15 mL centrifuge tube containing 10 mL of digestive juice and incubated in a 37 °C water bath for 1 h before adding 1 mL of fetal bovine serum to stop the digestion. Each cell suspension was mixed and transferred to another centrifuge tube before adding 5 mL of pure minimal essential culture media. The tubes were inverted to release more cells and these were also transferred to centrifuge tubes containing cell suspensions. The cell suspensions were centrifuged for 5 min at 1200 revolutions/min and the supernatants were removed. A total of 5 mL of Dulbecco's modified Eagle medium/F12 complete medium was added to the pellets at the bottom of each centrifuge tube, and cell suspensions were transferred to a 10 cm dish. The cells were incubated for 1–2 h at 37 °C in 5% CO2 to remove foreign cells. The cell suspension in each Petri dish was transferred to a centrifuge tube, centrifuged at 1200 revolutions/min for 5 min, and then cultured in a cell incubator with 5 mL of bronchial epithelial cell growth medium (Lonza, Walkersville, MD, USA) at 37 °C in 5% CO2.

At 80–90% confluence, the cells were stimulated for various durations using recombinant 100 ng/mL HNE (Merck Millipore, Burlington, MA, USA). Cell pellets were used for qRT-PCR analyses and cell supernatants were collected for analyses using ELISAs. Primary HNECs were treated with 100 ng/mL HNE in the presence or absence of 10 nM bafilomycin A1 (Sigma-Aldrich), or 10 μM SP600125 (Sigma-Aldrich) for 24 h.

The small interfering RNAs (siRNAs) for the human ATG5, BECN1, and c-JUN genes were obtained from Genechem (Shanghai, China). All siRNAs were transfected into cells using Enhanced Infection Solution (Genechem). After 72 h of transfection, cells were treated with HNE, as described above.

2.6. Transmission electron microscopy

For transmission electron microscopy (TEM), primary HNECs were fixed using 2.5% glutaraldehyde in PBS for 24 h after experimental manipulation. The cells were washed in PBS, post-fixed in 1% osmium tetroxide, and stained with 4% uranyl acetate. The samples were embedded in embedding medium after being dehydrated. Ultrathin sections were stained with uranyl acetate and lead citrate. Images were obtained at 80 kv using a HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

2.7. Western blotting

Western blotting was performed in accordance with the method described by Luo et al. [5]. Total protein was extracted from the isolated cells in 100 μL of radioimmunoprecipitation assay lysis buffer. The protein concentration in each supernatant was determined using the bicinchoninic acid assay method. Samples containing 20 μg of protein were boiled and subjected to sodium dodecyl sulfate- polyacrylamide gel electrophoresis in 8% Tris-glycine gels. The protein was electrophoretically transferred to polyvinylidene fluoride membranes, which were incubated for 1 h at room temperature in 5% fat-free skimmed milk in Tris buffered saline containing 0.05% Tween-20. Membranes were incubated overnight at 4 °C with diluted (1:1000) rabbit anti-human JNK (Abcam), rabbit anti-human c-Jun (Abcam), rabbit anti-human P-JNK (CST, Boston, MA, USA), rabbit anti-human p-c-Jun (CST), and β-actin polyclonal antibodies (Proteintech, Wuhan, China). Membranes were then washed and incubated in goat anti-rabbit antibodies (Zsjqbio, Beijing, China) for 1 h. Finally, membranes were washed three times with Tris buffered saline-Tween and exposed in a darkroom. β-actin was used as the protein loading control.

2.8. Statistical analysis

Statistical analyses were performed using SPSS software (ver. 22.0; SPSS, Inc., Chicago, IL, USA). Expression data are presented as dot plots with medians and interquartile ranges. For the in vitro experiments, cell culture data are presented as means ± standard error of the mean. Data were analyzed with GraphPad Prism software (ver. 6.0; GraphPad Software, Inc., San Diego, CA, USA). Differences between two groups were identified using Student's *t*-tests. A p-value < 0.05 was considered statistically significant.

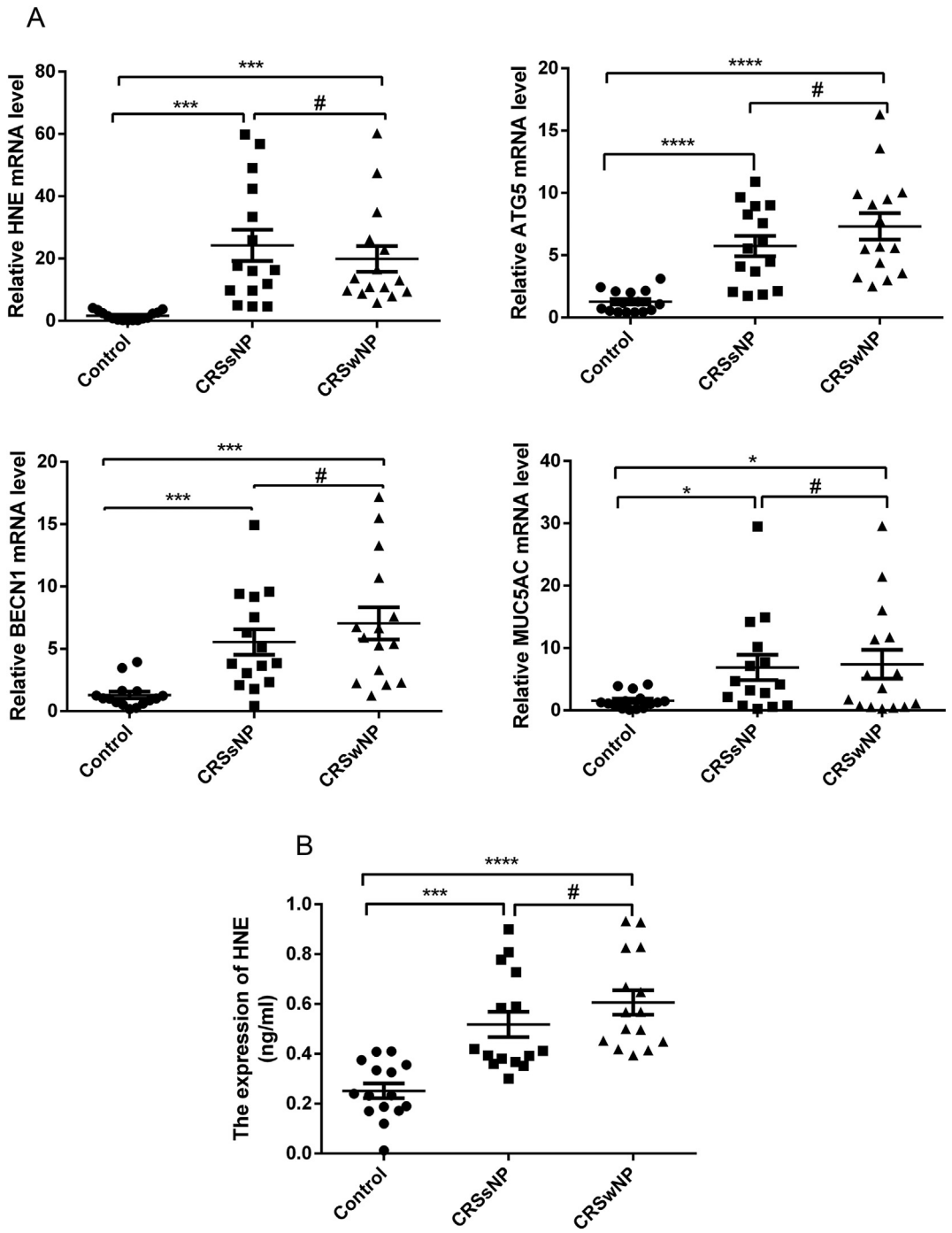

Fig. 1. Expression of autophagic-related genes(ATG5,BECN1), human neutrophil elastase, and MUC5AC in sinonasal tissue.
(A) Quantitative real-time polymerase chain reaction shows increased expression of autophagy-related gene 5 (*ATG5*), Beclin-1(*BECN1*), human neutrophil elastase (*HNE*) and mucin (*MUC*)*5 AC* in sinonasal tissuel from patients with chronic rhinosinusitis (CRS). (B) Enzyme-linked immunosorbent assay (ELISA) shows increased expression of HNE proteins in sinonasal tissue from patients with CRS. Data are presented as dot plots with medians and interquartile ranges.
Abbreviations: CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps. $*p < 0.05$; $***p < 0.001$; $****p < 0.0001$; $\#p > 0.05$.

3. Results

3.1. Upregulation of HNE and MUC5AC and activation of JNK–AP-1 signaling in nasal epithelial cells from patients with CRS despite induction of autophagy

We investigated autophagy and MUC secretion in tissues from patients with CRS. QRT-PCR analyses showed that HNE, MUC5AC, and the autophagic genes BECN1 and ATG5 were upregulated in tissue from patients with CRSsNP or CRSwNP compared to nasal tissue from control subjects (Fig. 1A).Enzyme-linked immunosorbent assay (ELISA) shows significantly increased expression of HNE proteins in sinonasal tissue from patients with CRSsNP or CRSwNP (Fig. 1B). We used immunohistochemical methods to identify cellular components with increased HNE, autophagic protein, JNK, c-Jun and MUC5AC expression in tissues from patients with CRSsNP or CRSwNP (Figs. 2–4A). We found that although some immunoreactivity was visible in cells infiltrating the lamina propria in patients with CRS, HNE, JNK, c-Jun,

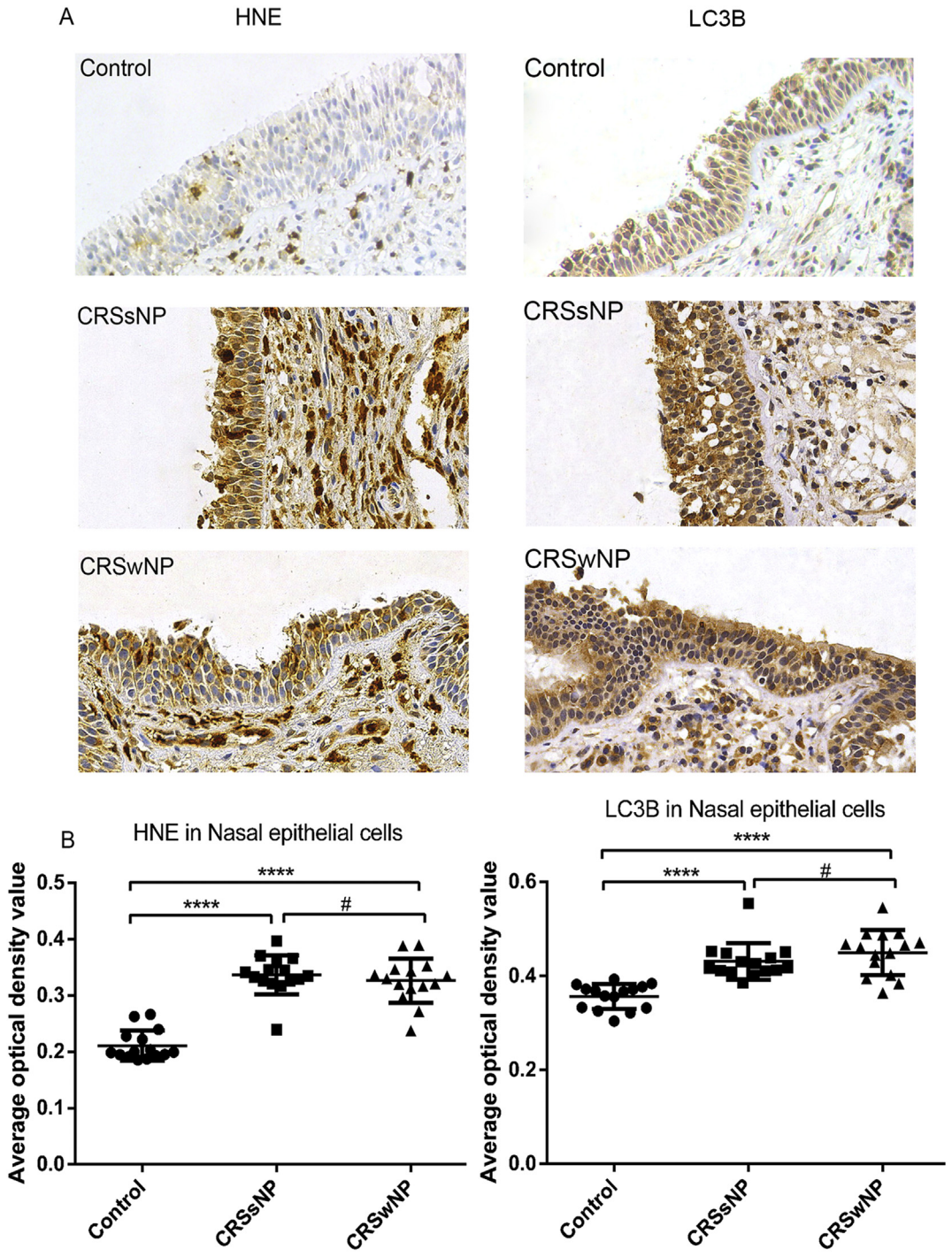

Fig. 2. Histological studies show increased expression of human neutrophil elastase and autophagic proteins in HNECs from patients with CRS.
Immunohistochemical staining demonstrates increased expression of light chain (LC)3B and HNE proteins in HNECs from patients with CRSsNP or CRSwNP compared to those from control subjects. Images are representative microphotographs showing immunohistochemically stained tissue sections. All sections were observed by light microscopy at 400× Magnification.
Average optical density analysis of the immunohistochemical results show that the levels of HNE and LC3B-II proteins were all increased in HNECs from patients with CRSsNP or CRSwNP compared to those from control subjects. Data are presented as dot plots with medians and interquartile ranges. $^{****}p < 0.0001$; $\#p > 0.05$.

MUC5AC and the autophagic proteins, including LC3B, were all predominantly expressed in nasal epithelial cells. HNE, JNK, c-Jun, MUC5AC and Autophagic protein expression levels increased significantly in nasal epithelial cells from patients with CRSsNP or CRSwNP compared to control subjects (Figs. 2–4). Average optical density analysis of the immunohistochemical results show that the levels of HNE,LC3B-II, JNK, c-Jun and MUC5AC proteins were all increased in nasal epithelial cells from patients with CRSsNP or CRSwNP compared to those from control subjects (Figs. 2–4B).These results suggest that nasal epithelial cells are the major cell type showing altered autophagic and MUC5AC secretion in patients with CRS.

3.2. HNE induces MUC5AC expression in primary HNECs

To investigate the role of autophagy in mucus production, we treated primary HNECs with HNE. As expected, HNE significantly

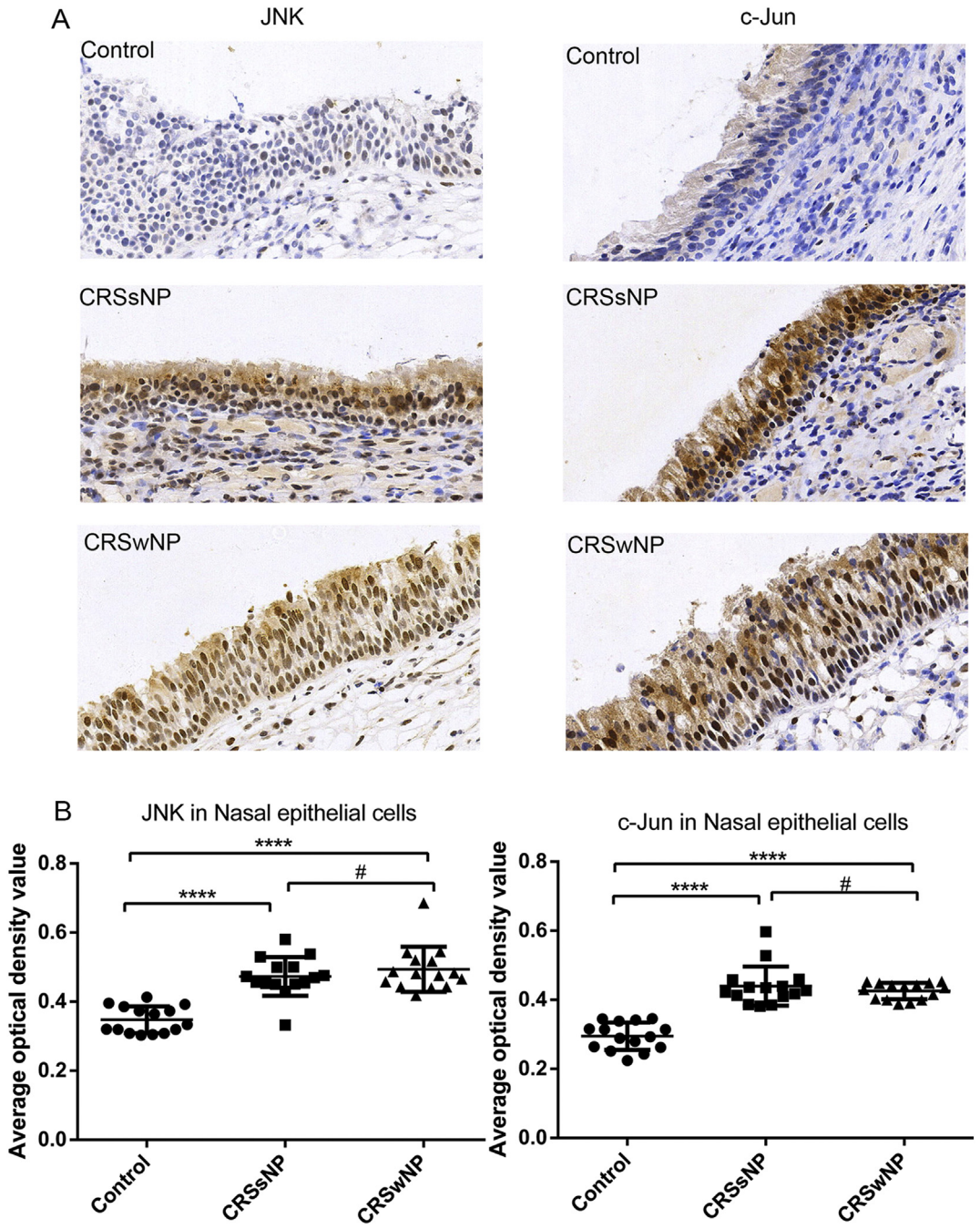

Fig. 3. Histological studies show increased expression of c-Jun N-terminal kinase (JNK) and c-Jun in HNECs from patients with CRS.
(A)Immunohistochemical staining demonstrates increased expression of JNK and c-Jun in HNECs from patients with CRSsNP or CRSwNP compared to those from control subjects. Images are representative microphotographs showing immunohistochemically stained tissue sections. All sections were observed by light microscopy at 400× Magnification.
(B)Average optical density analysis of the immunohistochemical results show that the levels of JNK and c-Jun were all increased in HNECs from patients with CRSsNP or CRSwNP compared to those from control subjects. Data are presented as dot plots with medians and interquartile ranges. $^{****}p < 0.0001$; $\#p > 0.05$.

upregulated MUC5AC expression in primary HNECs. Just like our previous research [5] HNE-induced MUC5AC mRNA expression in a dose-dependent and time-dependent manner. The TEM images showed autophagy in primary HNECs that had been treated with HNE (Fig. 5A). Bafilomycin A1 is an inhibitor of autophagosome fusion with lysosomes, blocking the process of autophagosome and lysosomal fusion. Therefore, after the use of bafilomycin A1, there will be an increase in autophagosomes and a decrease in the number of Autophagy lysosome. When Bafilomycin A1(Baf A1) stimulated primary HNECs, LC3B-II(a key marker for autophagosome) increased. Treatment of HNE with a lysosome inhibitor bafilomycin A1 (Baf A1) further enhanced the induction of LC3B-II (Fig. 5B), suggesting that the increased levels of LC3BII by HNE treatment were indeed induced, rather than due to an impaired autophagosome-lysosome fusion.

3.3. Autophagy is required for HNE-induced expression of MUC5AC in primary HNECs

The induction of autophagy can be either cytoprotective or deleterious. Because HNE can induce autophagy in lung epithelial cells

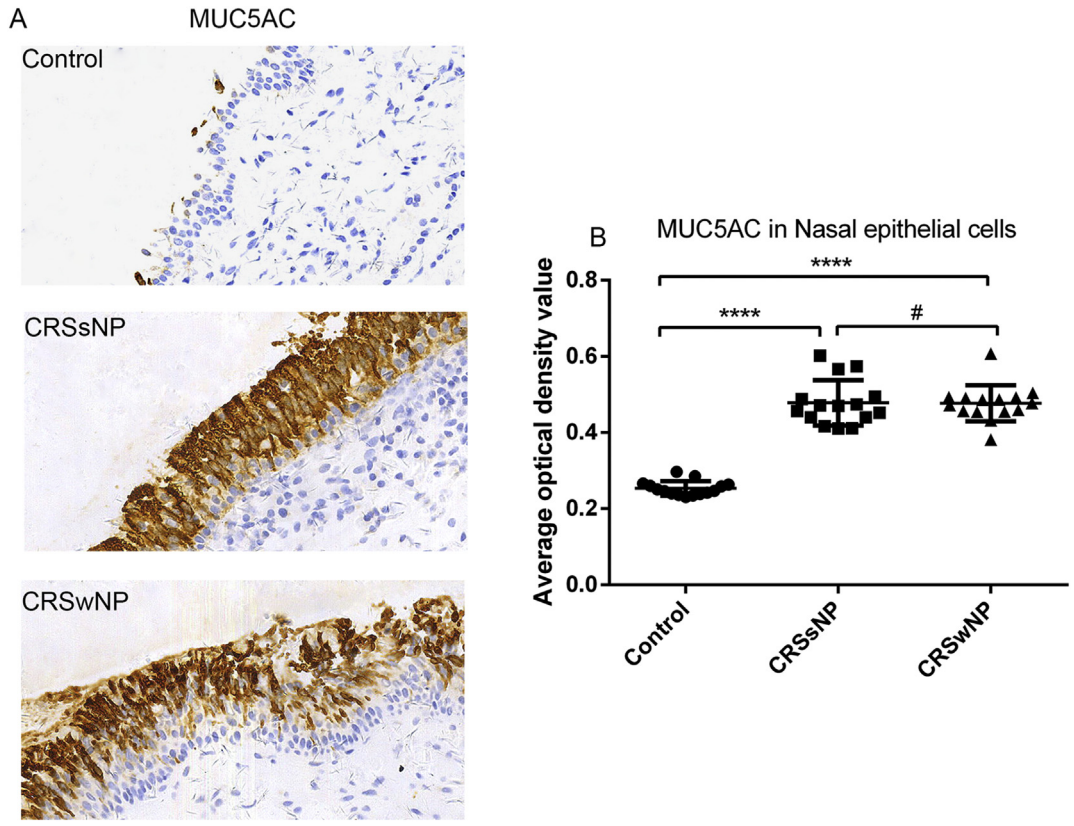

Fig. 4. Histological studies show increased expression of MUC5AC in HNECs from patients with CRS.
Immunohistochemical staining demonstrates increased expression of MUC5AC in HNECs from patients with CRSsNP or CRSwNP compared to those from control subjects. Images are representative microphotographs showing immunohistochemically stained tissue sections. All sections were observed by light microscopy at 400× Magnification.
Average optical density analysis of the immunohistochemical results show that the levels of MUC5AC were all increased in HNECs from patients with CRSsNP or CRSwNP compared to those from control subjects. Data are presented as dot plots with medians and interquartile ranges. ****$p < 0.0001$; #$p > 0.05$.

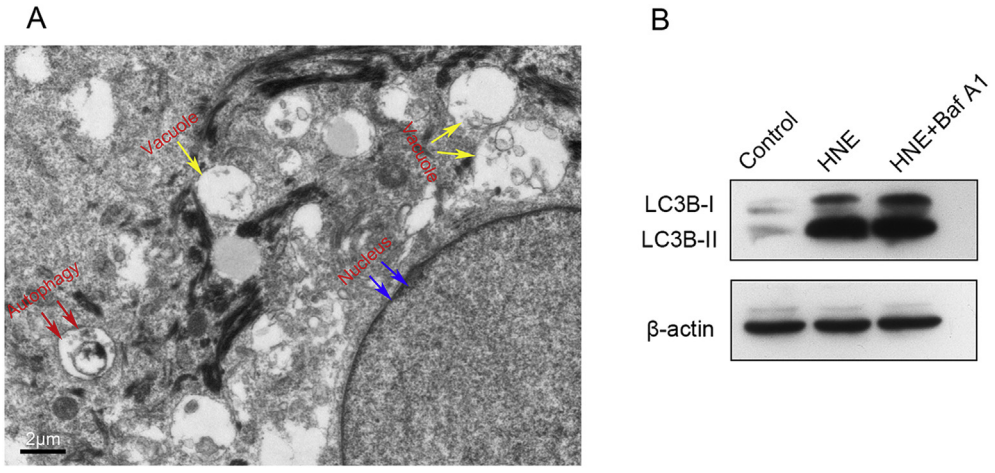

Fig. 5. Transmission electron microscopy images showing autophagy in primary HNECs treated with 10 nM HNE for 24 h.
(A) Representative transmission electron microscopy image of primary HNECs treated with HNE. (B) Western blotting analysis of the induction of LC3B-II by HNE in primary HNECs. Treatments were for 24 h with 10 nM HNE and/or 10 nM bafilomycin A1.

[29], and induction of autophagy triggers MUC expression, we investigated the possible role of autophagy in HNE-induced MUC5AC expression in primary HNECs. HNE markedly increased MUC5AC mRNA and secreted protein levels in primary HNECs [5]. We also tested siRNAs that targeted various ATG5 and BECN1. The knockdown effects of these siRNAs are shown in Fig. 6A. Interestingly, HNE-induced MUC5AC mRNA expression (Fig. 6B) and protein levels (Fig. 6C) were markedly attenuated in primary HNECs transfected with these autophagy-related siRNAs. This suggests a deleterious effect of autophagy in mediating HNE-induced inflammation in primary HNECs.

Mucus hypersecretion by primary HNECs is a key feature of chronic nasal-sinus diseases. This results in nasal obstruction and is a significant cause of morbidity. However, little is known about the role of HNE in mucus production. > 20 MUC genes have been identified, and MUC5AC is one of the most widely expressed MUCs in the airway. It is also frequently induced by pathological conditions [30]. Interestingly, we found that HNE significantly induced MUC5AC mRNA and protein expression in HNECs [5]. Elevated MUC5AC expression was also effectively attenuated by knockdown of BECN1 or ATG5 expression (Figs. 6B, C), indicating that autophagy plays an essential role in HNE-

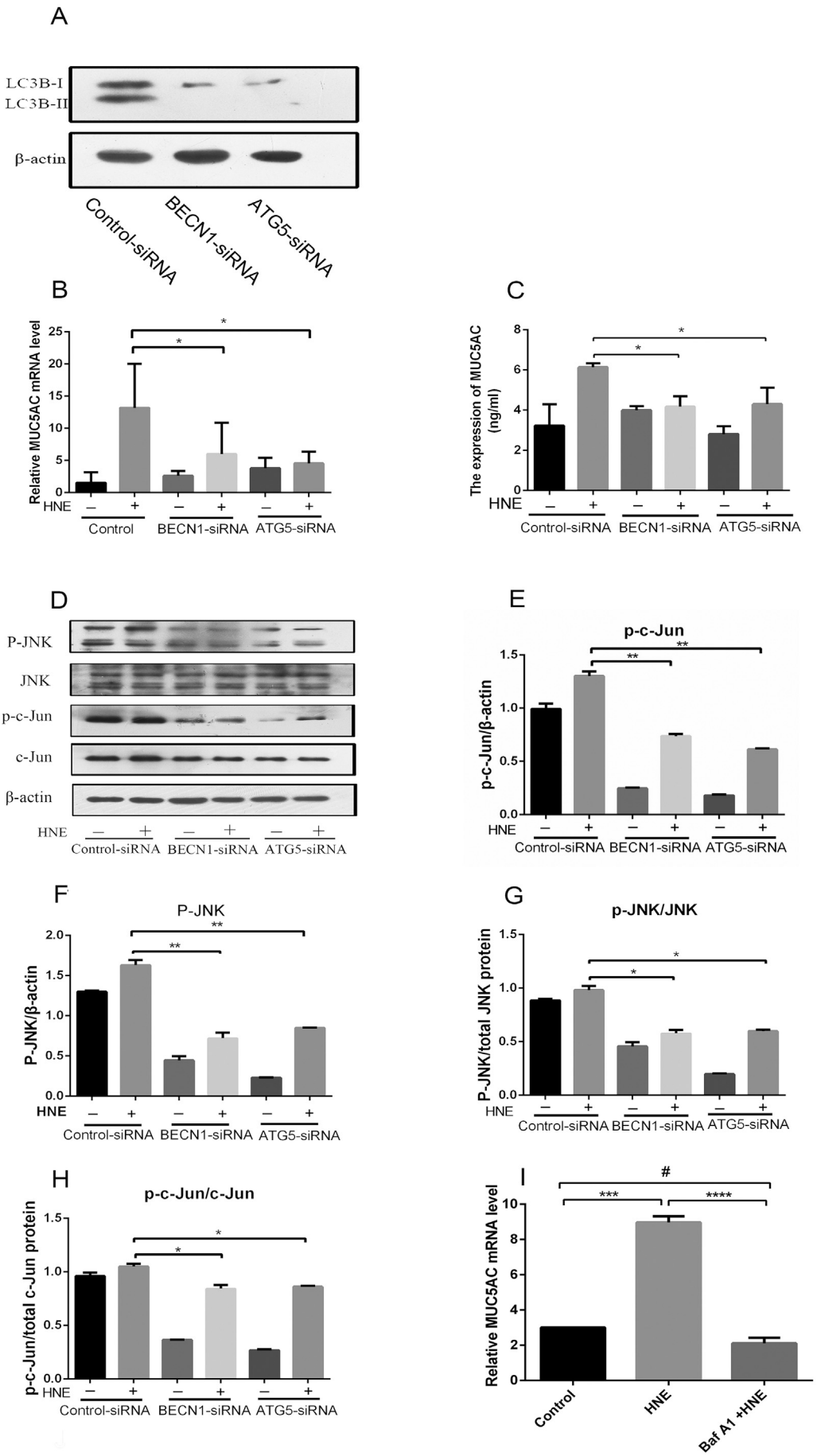

Fig. 6. Autophagy in HNE-induced MUC5AC and JNK–AP-1 signaling.
(A–H): Primary HNECs were transfected with the small interfering RNAs (siRNAs) shown for 72 h, and then treated with 10 nM HNE for another 24 h. (A) Knockdown effects of siRNAs on autophagy-related targeted proteins. MUC5AC mRNA expression (B) and protein levels (C, ELISA) were analyzed. (D–H): The expression levels of p-JNK, JNK, p-c-JUN, and c-JUN were analyzed using western blotting. Primary HNECs were treated with the lysosomal inhibitor bafilomycin A1 (10 nM) together with HNE (10 nM) for 24 h. (I) Relative *MUC5AC* mRNA expression normalized to β-actin. The results shown are from at least three independent experiments. All data are expressed as means ± standard error of the mean.
Abbreviation: AP-1, activator protein-1. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

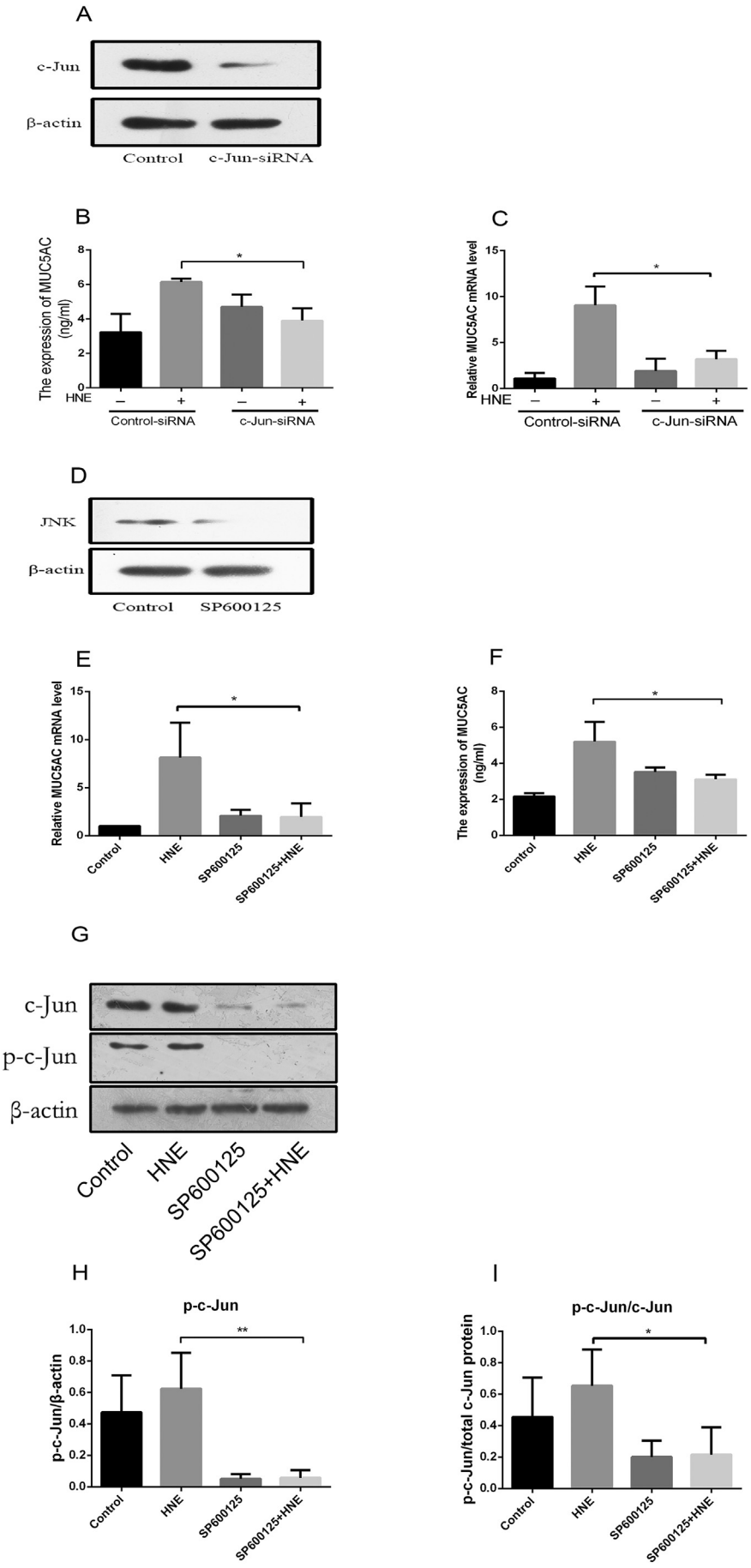

Fig. 7. Role of the JNK–AP-1 pathway in mediating HNE-induced MUC5AC expression.
(A) The knockdown effect of c-JUN-siRNA. (B−C): The effect of c-JUN-siRNA on HNE-induced MUC5AC mRNA expression (C) and protein levels (B, ELISA). After siRNA transfection for 72 h, cells were incubated with 10 nM HNE for another 24 h. (D) The effect of inhibition using SP600125. (E and F): The effect of the JNK inhibitor SP600125 on HNE-induced MUC5AC mRNA expression (E) and protein levels (F, ELISA). The expression of p-c-JUN and c-JUN was analyzed using western blotting (G–I). The results shown are from at least three independent experiments. All data are expressed as means ± standard error of the mean. $^{*}p < 0.05$; $^{**}p < 0.01$.

induced MUC production in primary HNECs. We also investigated the effects of bafilomycin A1 on HNE-induced MUC production. Surprisingly, treatment with bafilomycin A1 markedly inhibited the expression of MUC5AC (Fig. 6I). Lysosomal inhibitors are widely used to inhibit autophagosome-lysosome fusion. In addition, bafilomycin A1 almost completely abolished HNE-induced MUC5AC expression.

3.4. Autophagy regulates HNE-induced mucus production via AP-1

Some transcription factors, such as ERK1/2 and AP-1, may regulate MUC5AC expression in primary HNECs [31]. HNE stimulates autophagy in HNECs and was associated with the activation of 5' AMP-activated protein kinase and the inhibition of the serine/threonine kinase mTOR [21]. We investigated whether autophagy regulates HNE-induced MUC5AC expression via one of these transcription factors. Knockdown of c-JUN by siRNA (Fig. 7A) or inhibition of AP-1 activity by SP600125 (Fig. 7D) suppressed HNE-induced MUC5AC expression (Fig. 7B-I). These data suggest that HNE-induced MUC5AC expression in primary HNECs is mediated, at least partially, by induced the phosphorylation of JNK and c-Jun.

3.5. Activation of the JNK-AP-1 pathway by autophagy and inhibition of HNE-induced expression of MUC5AC in primary HNECs

These results encouraged us to investigate the mechanisms mediating the effects of autophagy on HNE-induced expression of MUC5AC. Because JNK and AP-1 mediate inflammatory responses and mucus production in response to airway injury, we examined the roles of these proteins in HNE-induced damage to the nasal epithelial cells. Inhibition of the AP-1 pathway by SP600125 or c-Jun-siRNA markedly attenuated the HNE-induced upregulation of MUC5AC (Fig. 7). These data suggest that the JNK–AP-1 pathway regulates HNE-induced expression of MUC5AC in primary HNECs. However, BECN1-siRNA/ATG5-siRNA markedly decreased the phosphorylation of JNK and c-Jun (Fig. 6D–H), suggesting that autophagy is required for activation of the JNK–AP-1 pathway.

4. Discussion

In this study, we have clearly demonstrated that HNE triggers autophagy in primary HNECs, and that autophagy is essential for HNE-induced mucus hypersecretion via the JNK–AP-1 pathway. We have also shown that autophagy regulates MUC5AC expression partially by modulating JNK signaling and the transcription factor AP-1 (Fig. 8).

Recent research suggests that autophagy is essential for the production of pro-inflammatory cytokines [32,33]. Lipopolysaccharides (LPSs) reportedly induce autophagy in HNECs [21]; however, there is little evidence that HNE is secreted by autophagosomes. However, we showed that HNE stimulates the appearance of autophagosomes in primary HNECs (Fig. 5A).

Autophagy in HNECs and the upper respiratory tract cell damage caused by various diseases may be deleterious or cytoprotective, probably depending on the presence of different stimuli and pathogens. For example, in hyperoxia-induced airway epithelial damage, LC3B-siRNA promotes hyperoxia-induced cell death, whereas overexpression of LC3B confers cytoprotection [34]. However, LPSs stimulate autophagy in HNECs in a similar way to CRS pathogenesis. Overexpression of LC3B results in cellular inflammation [13]. H5N1 infection also induces autophagic cell death in lung epithelial cells, and inhibiting autophagy reduces epithelial cell death [35,36], decreases the production of pro-inflammatory cytokines and chemokines [36], and ameliorates H5N1-induced acute lung injury and mortality [37]. Overexpression of LC3Bs damages cells. Our results are consistent with those studies, showing that autophagy stimulated HNE-induced epithelial secretion of MUCs. Inhibiting autophagy attenuated MUC5AC production in HNECs. Therefore, targeting autophagy inhibition in airway epithelial cells or HNECs might be an effective treatment for COPD [38,39], influenza infection [37], LPS-induced airway inflammation [21], HNE-induced airway inflammation, and MUC secretion.

Mucus hypersecretion is another important feature of chronic airway diseases. The histological features of CRS lesions in the sinus mucosa suggest the infiltration of large numbers of cells, including neutrophils, and the role of neutrophils in CRS has become a focus of recent research [40–42]. Neutrophils secrete inflammatory mediators. One of these, HNE, has a significant effect on the immune status of the nasal sinus and the entire airway mucosa. In vitro or in vivo experiments using respiratory epithelial cells have shown that metaplasia and the proliferation of goblet cells can occur, inducing increases in MUC5AC expression and secretion [43,44]. Studies have also investigated the role of HNE in mucus production. For example, Scutellarin attenuates human-neutrophil-elastase-induced mucus production by inhibiting the PKC-ERK signaling pathway [45]. In human bronchial epithelial cells and human lung cancer cells, HNE induces MUC5AC expression [46,47]. Another study showed that HNE induces MUC5AC overexpression in patients with CRS via tumor necrosis factor-α converting enzyme, which subsequently produces excessive MUC in patients with CRSsNP or CRSwNP [7]. Our data clearly demonstrate that HNE is essential for inducing mucus hypersecretion in HNECs.

To the best of our knowledge, this is the first study to demonstrate a role for autophagy in the regulation of HNE-induced MUC5AC expression in the human nasal epithelium. We showed that HNE treatment induces autophagy in primary HNECs and could induce MUC5AC expression. In addition, the lysosomal inhibitor bafilomycin A1 completely abolished HNE-induced MUC production, suggesting that the autophagic lysosomal degradation process is necessary for subsequent production of MUC5AC.

Although this study clarified the role of autophagy in regulating MUC gene transcription, two recent studies also investigated the mucus secretion-modulating function of autophagy. Patel et al. showed that autophagic proteins can control mucus secretion in colonic goblet cells via reactive oxygen species [48]. In addition, IL-13 can activate autophagy to regulate mucus secretion in airway epithelial cells [49]. In both studies, inhibiting autophagy resulted in increased mucus accumulation, probably due to decreased mucus secretion. However, our in vitro data demonstrated that deficiencies in autophagy reduced the levels of MU5AC mRNA, and eventually decreased MUC5AC protein and mRNA levels in primary HNECs. However, these studies suggest that autophagy not only regulates MUC transcription, but also orchestrates mucus secretion, both of which are crucial in disease pathogenesis.

Our previous studies, and those of other authors, have demonstrated that HNE can induce MUC5AC secretion in primary HNECs [7], although the signaling pathway that mediates this process is not completely understood. Several proteins have been implicated in MUC5AC expression, depending on cell type and stimuli. This study revealed that the JNK–AP-1 pathway was critical for HNE-induced MUC5AC expression in primary HNECs. However, the detailed mechanisms by which autophagy regulates JNK phosphorylation remain unclear. The autophagy machinery probably does not directly modulate JNK phosphorylation; it is more likely that downstream molecules and signaling events activate JNK.

In summary, this study clearly demonstrates that autophagy is essential for HNE-induced activation of the JNK–AP-1 signaling pathway, and this subsequently promotes excessive MUC production in patients with CRSsNP or CRSwNP. Our findings imply that autophagy may be targeted as a selective therapeutic strategy to treat MUC hypersecretion in patients with CRSsNP or CRSwNP.

Conflicts of interest

All authors declare no conflict of interest.

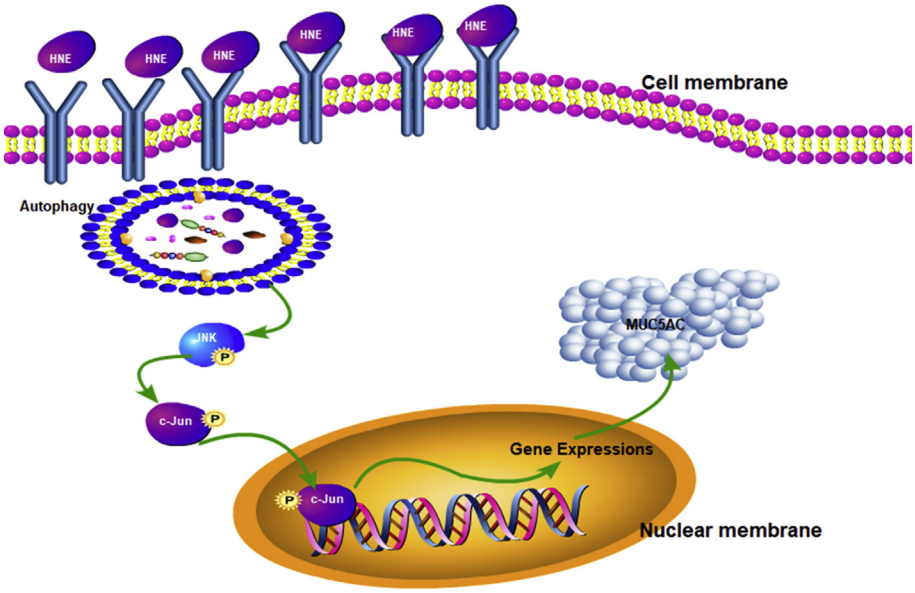

Fig. 8. Diagram showing how autophagy regulates HNE-induced MUC5AC expression in HNECs.
Exposure to HNE stimulates autophagy, which regulates MUC5AC expression by modulating JNK signaling and the transcription factor AP-1.

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