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Heat shock protein 70 is induced by pepsin via MAPK signaling in human nasal epithelial cells

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Abstract

Background Recent studies have shown that laryngopharyngeal reflux is associated with chronic rhinosinusitis. Pepsin may be a key factor involved in the injury of nasal mucosal epithelial cells, but the pathogenesis remains unclear. We are to investigate whether a mitogen-activated protein kinase (MAPK) pathway regulates heat shock protein 70 (HSP70) expression in primary cultures of human nasal epithelial cells (HNEpCs) in response to pepsin stimulation.

Methods HSP70 protein expression levels in HNEpCs were estimated by Western blot analysis after treatment with pepsin. MAPK pathway activity levels were also evaluated to elucidate the mechanism underlying the effects of pepsin on HSP70 in HNEpCs. Inhibitors of signaling pathways were used to determine the contribution of MAPKs in HSP70 response after pepsin stimulation. Cellular apoptosis and cell viability in HNEpCs after treatment with pepsin were measured.

Results The expression of HSP70 increased after stimulation with pepsin and decreased after the removal of pepsin. Pepsin induced activation of p38, extracellular signal-regulated kinase 1/2, and c-Jun N-terminal kinase (JNK) 1/2. Inhibition of JNK1/2 reduced HSP70 expression in HNEpCs. The apoptosis in HNEpCs at 12 h after treatment with pepsin at pH 7.0 increased significantly when compared with the control and pH 7.0 groups. Cell viability decreased following exposure to pepsin at pH 7.0.

Conclusion Pepsin, even under neutral pH 7.0, increases the expression of HSP70 in HNEpCs by activating the JNK/MAPK signaling pathway. Increased HSP70 may be the protective mechanism when pepsin presents in the other parts of the body.

Keywords Laryngopharyngeal reflux · Pepsin · Chronic rhinosinusitis · Heat shock protein 70 · Mitogen-activated protein kinase

Introduction

Chronic rhinosinusitis (CRS) is one of the most common chronic diseases, with an overall prevalence of approximately 12% [1]. Many studies have indicated that laryngopharyngeal reflux (LPR) is a risk factor for CRS [2, 3]. LPR is an upper respiratory tract disease that results from the reflux of gastric contents to the pharynx, larynx, oral cavity, nasal cavity, or trachea. The symptoms and injury associated with LPR may be not only caused by the reflux of acidic gastric contents (pH < 4.0) but also associated with non-acid reflux (pH 4.0–7.0) [4, 5]. It has been suggested that LPR causes mucosal damage and inflammatory and mucociliary dysfunction, which may underlie the pathogenesis of CRS. Most studies considered that pepsin in the reflux of gastric contents may play a vital role in this process [6].

Heat shock proteins (HSPs) are a large family of proteins synthesized under external stimulation and are involved in protective mechanisms. Chemical or physical stimulation leads to increased expression of HSPs, which can prevent cell damage. The protective effects of HSPs are related to their functions as molecular chaperones [7, 8]. As a member of HSP, HSP70 is thought to inhibit

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apoptosis, stimulates cytokine production and autophagy, and promotes epithelial cell growth and proliferation [9, 10].

HSP70 is associated with apoptosis after myocardial ischemia and reperfusion, neuropathy, diabetes, epilepsy, and other chronic diseases [11–13], and it plays an important role in the pathophysiological process of CRS [14]. The previous studies have shown that the expression of HSP70 in laryngeal mucosa is related to LPR [15]. We previously demonstrated that HSP70 levels were significantly increased in pepsin-positive turbinate mucosa compared with control mucosa in CRS patients [16]. However, the signaling pathways involved in HSP70 regulation need further study.

The mitogen-activated protein kinase (MAPK) pathway is an evolutionarily conserved signaling pathway existing in unicellular organisms to humans [17]. The three most common MAPK pathways discovered, thus far, include extracellular-regulated protein kinase (ERK) 1/2, p38 and c-Jun N-terminal kinase (JNK) 1/2 [18–20]. Most signals that activate ERK pathways are closely related to the activation of receptors or ion channels, which ultimately leads to cell proliferation and transformation [21, 22]. JNK and p38 affect cell survival and death under toxic stimuli such as calcium ions, heat stress, and reactive oxygen species [23–25].

The MAPK pathway plays roles in both protection and injury with its cell-protective effects. Many studies have demonstrated that activation of MAPK signaling pathways increases the expression of HSP70, thereby protecting cells against stress [26, 27]. In the present study, we investigated the expression of HSP70 and MAPK pathway members, and the relationships among them, by stimulating human nasal epithelial cells (HNEpCs) with pepsin, which may contribute to the pathophysiological mechanisms of reflux-induced CRS.

Materials and methods

Reagents

Airway Epithelial Cell Medium and Growth Medium SupplementMix were purchased from PromoCell (Heidelberg, Germany). Porcine pepsin was purchased from Sigma-Aldrich (St. Louis, MO, USA). SB-203580, PD-098059, and SP-600125 were obtained from Medchem Express (Monmouth Junction, NJ, USA). Anti-phospho-p44/42 (Thr202/Tyr204), anti-p44/42, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-SAPK/JNK, anti-phospho-p38 (Thr180/Tyr182), and anti-p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-HSP70 was obtained from Abcam (Cambridge, UK).

Cell culture and treatments

HNEpCs were obtained from PromoCell. Cells were grown in an appropriate volume of PromoCell Growth Medium supplemented with Growth Medium SupplementMix. Cells were cultured at 37 °C in an incubator with humidified air and 5% CO_2. All experiments were carried out using primary HNEpC cultures between passages 8 and 12. After 24–48 h of culture, cells were serum-starved overnight before the treatments.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS), and incubated in 0.1% Triton X-100/PBS for 10 min. The cells were incubated with the primary mouse monoclonal anti-HSP70 antibody (1:100, ab2787, Abcam) overnight at 4 °C, followed by secondary antibodies for 60 min at room temperature. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich), and stained cells were observed under a fluorescent microscope.

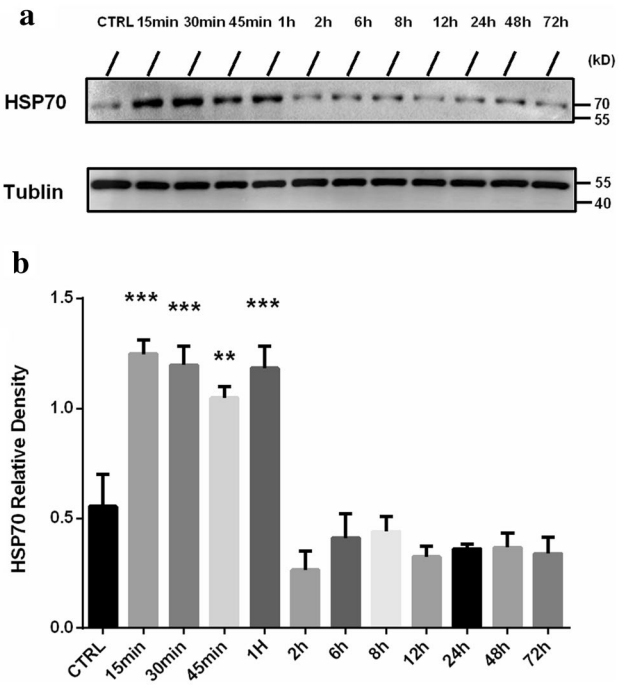

Fig. 1 Protein levels of heat shock protein 70 (HSP70) in human nasal epithelial cells (HNEpCs) after pepsin stimulation. **a** HSP70 and tubulin protein levels were measured in HNEpCs by Western blot analysis. **b** Expression of HSP70 was significantly higher in HNEpCs stimulated with pepsin than in the control cells (gray intensity analysis of Western blot results). Data represent means ± standard error of the mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

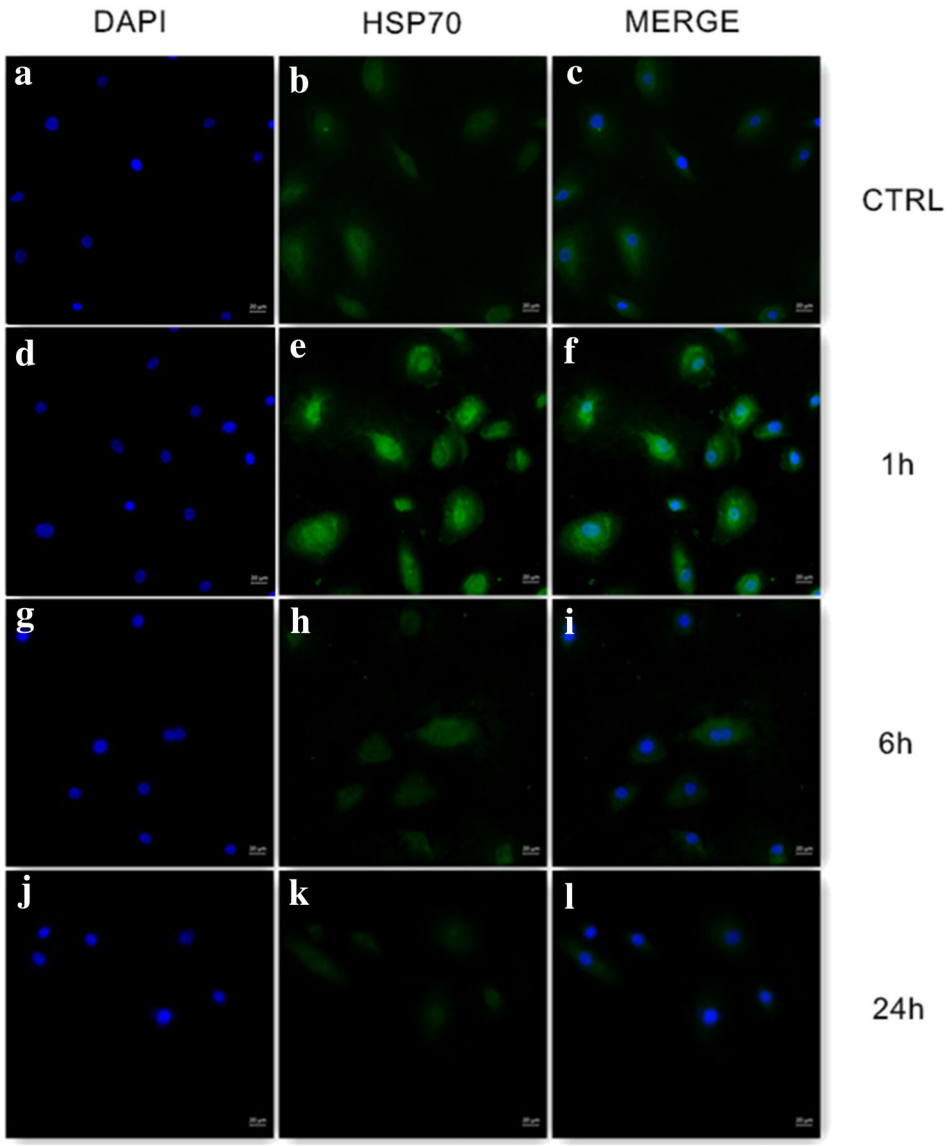

Fig. 2 Immunological characterization of HSP70 in HNEpCs. **a**–**c** Immunofluorescence staining of HSP70 (green) and staining of nuclei (blue) in HNEpCs before exposure to pepsin (0.1 mg/mL, pH 7.0). **d**–**f** Immunofluorescence staining of HSP70 and staining of nuclei in HNEpCs after exposure to pepsin for 1 h. **g**–**i** Immunofluorescence staining of HSP70 and staining of nuclei in HNEpCs at 6 h after exposure to pepsin. **j**–**l** Immunofluorescence staining of HSP70 and staining of nuclei in HNEpCs at 24 h after exposure to pepsin

Western blot analysis

HSP70, phospho-p44/42, p44/42, phospho-JNK, JNK, phospho-p38, and anti-p38 protein expression was assessed in HNEpCs after exposure to 0.1 mg/mL pepsin at pH 7.0 for 60 min, with or without the inhibitors SB-203580, PD-098059, and SP-600125. Cells were then incubated in Airway Epithelial Cell Medium for the indicated times at 37 °C. Proteins extracted from cells were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes and blocked in 5% skim milk in Tris-buffered saline containing Tween-20 (TBS-T; 0.02 Tris, 0.15 NaCl, 0.05% Tween-20, and pH 7.5) for 1 h, and then incubated overnight at 4 °C with anti-HSP70 (1:1000), anti-phospho-ERK1/2 (1:1000), anti-ERK1/2 (1:1000), anti-phospho-SAPK/JNK (1:1000), anti-SAPK/JNK (1:2000), anti-phospho-p38 (1:1000), or anti-p38 (1:1000) diluted in TBS-T with 5% skim milk. After three washes with TBS-T, the membranes were incubated with goat anti-rabbit and anti-mouse secondary antibodies (1:5000, Zeng BioScience, Chengdu, China) in TBS-T with 5% skim milk for 1 h. The signal was obtained using enhanced chemiluminescence agents (Immobilon Western, SuperECL Plus, Millipore, Billerica, MA, USA).

Cell viability assay

HNEpC viability was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cells (3×10^3) were incubated in 96-well plates and treated according to the manufacturer's instructions. Absorbance was measured on a microplate reader (Gene Company Limited, Hong Kong, China) at 450 nm.

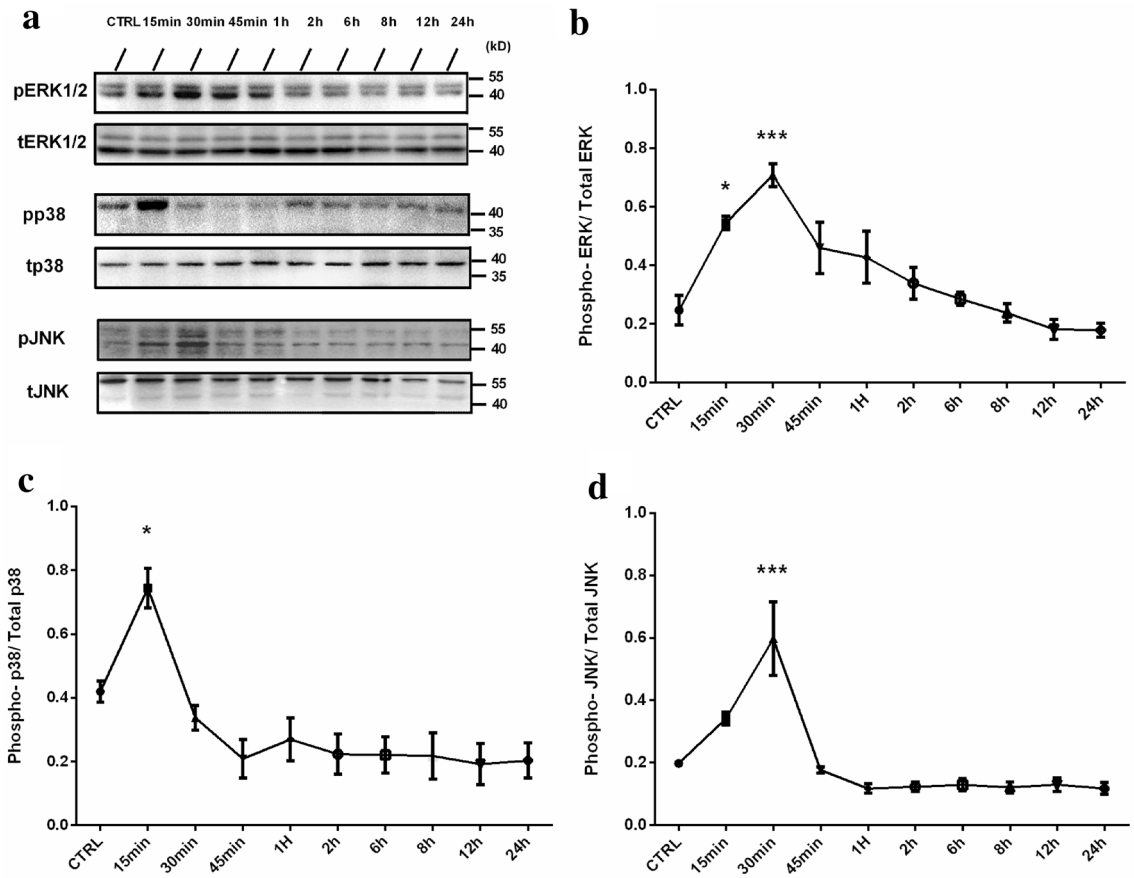

Fig. 3 MAPK phosphorylation levels in HNEpCs after exposure to pepsin (0.1 mg/mL, pH 7.0) were analyzed by Western blotting. **a** Phosphorylated and total ERK1/2, p38, and JNK1/2 levels were measured in HNEpCs by Western blot analysis. **b–d** ERK1/2, p38, and JNK1/2 MAPK pathway activities are expressed as the ratio between the phosphorylated and total forms. Data represent means ± standard error of the mean. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

Apoptosis and necrosis assays

The annexin V-FITC apoptosis assay (Keygen Biotech, Nanjing, China) was used for detection of cell death. Nasal epithelial cells were digested with 0.25% trypsin without EDTA. Cells were resuspended in binding buffer containing 5 µL annexin V–FITC and 5 µL propidium iodide, and incubated for 15 min at 37 °C in the dark. Stained cells were then analyzed by flow cytometry.

Statistical analysis

SPSS software (ver. 22; IBM SPSS, Armonk, NY, USA) was used to conduct all statistical analyses. Each experiment in this study was performed in triplicate. When a homogeneity test for variance was completed, all sample groups were compared using one-way analysis of variance followed by Turkey's multiple comparison tests. *P* values of 0.05 (*), 0.01 (**), and 0.001 (***) were considered significant.

Results

HSP70 protein expression in HNEpCs

HSP70 protein expression in HNEpCs was measured by Western blot analysis before and after exposure to 0.1 mg/mL pepsin at pH 7.0 for 1 h. The expression of HSP70 in HNEpCs stimulated with pepsin was significantly higher than in the control cells. After stimulation, the expression of HSP70 protein decreased, and there was no significant difference between the two groups (Fig. 1). Immunofluorescence staining was used to confirm the expression of HSP70 in HNEpCs treated with pepsin. HSP70 was observed in the cytoplasm and nuclei of HNEpCs. As shown in Fig. 2, the presence of HSP70 in HNEpCs was the most pronounced at 1 h after pepsin stimulation, consistent with the Western blot analysis.

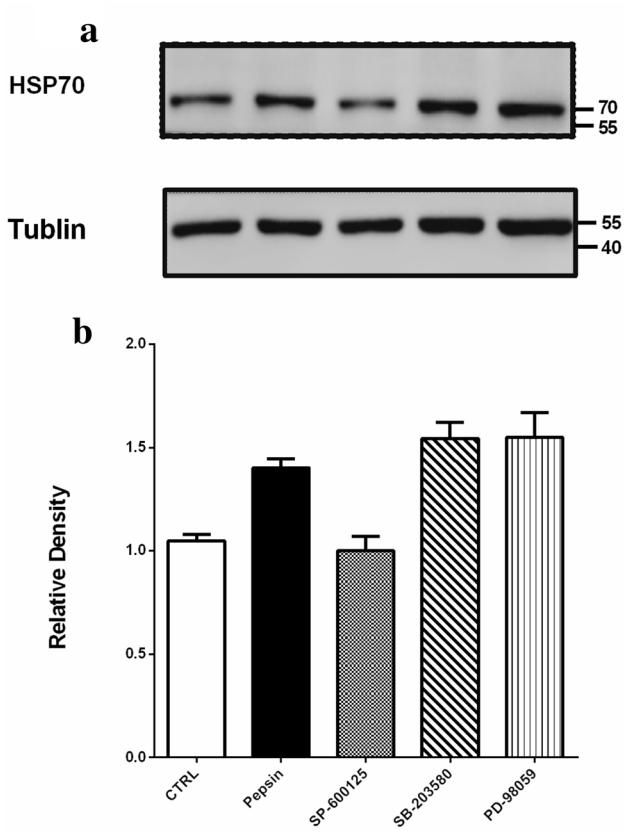

Fig. 4 Effects of MAPK inhibitors on HSP70 expression in HNEpCs, evaluated by Western blot analysis. **a** HSP70 expression measured by Western blot analysis in HNEpCs after exposure to pepsin (0.1 mg/mL, pH 7.0). **b** SP-600125 (specific inhibitor of JNK1/2) decreased HSP70 protein expression in HNEpCs after pepsin stimulation. However, PD-098059 (specific inhibitor of ERK1/2) and SB-203580 (specific inhibitor of p38) had no effect on HSP70 protein expression

MAPK phosphorylation in response to pepsin

As shown in Fig. 3, exposure to pepsin increased the phosphorylation levels of ERK1/2, p38 and JNK1/2 (MAPK pathway proteins). The level of phospho-ERK1/2 in HNEpCs increased significantly after stimulation, peaked at 30 min, and then began to decrease at 45 min, suggesting activation of ERK1/2 (Fig. 3b). The phosphorylation of p38 MAPK peaked at 15 min and then started to decrease at 30 min, suggesting activation of p38 (Fig. 3c). The level of phospho-JNK1/2 in HNEpCs was increased significantly at 30 min after pepsin stimulation, followed by a decrease at 45 min, suggesting activation of JNK1/2 (Fig. 3d).

Effect of inhibitors on HSP70 expression in HNEpCs

We determined the effect of MAPK inhibitors and HSP70 expression in pepsin-treated HNEpCs by Western blot analysis. As shown above, pepsin treatment led to an increase in HSP70 protein expression. PD-098059, SB-203580, and SP-600125 are specific inhibitors of ERK1/2, p38, and JNK1/2, respectively. SP-600125 decreased HSP70 protein expression in HNEpCs after pepsin stimulation. However, PD-098059 and SB-203580 had no effect on HSP70 protein expression. These results suggest that JNK1/2 activation plays a role in HSP70 expression in response to pepsin in HNEpCs (Fig. 4).

Cellular apoptosis

We determined the effect of pepsin on apoptosis in HNEpCs using the annexin V-FITC apoptosis assay (Fig. 5a, b). The apoptosis in HNEpCs at 12 h after treatment with pepsin at pH 7.0 was significantly increased compared with the control and pH 7.0 groups.

Cell viability

We assessed cell viability at 12, 24, and 48 h after treatment with pepsin at pH 7.0 using the Cell Counting Kit-8 assay. Figure 5c shows that cell viability decreased following exposure to pepsin at pH 7.0. There was no significant difference between HNEpCs treated with pepsin at pH 7.0 and the control. These results are consistent with the annexin V-FITC apoptosis assay results.

Discussion

Pepsinogen is a protease enzyme secreted by chief cells of the stomach, which convert into pepsin in an acidic environment. Activity and stability of pepsin are closely related to pH. Pepsin is active at pH <6.5 and inactive at pH ≥ 6.5. Pepsin can remain stable up to pH 8.0 and is reactivated when the pH is reduced [28]. Pepsin destroys the epithelial barrier by digesting intercellular junction proteins, inducing an oxidative stress response, damaging mitochondria, and participating in the inflammatory response. Adhami et al. [29] showed that pepsin damaged laryngeal tissue in an experimental canine model. Johnston et al. reported that laryngeal epithelial cells took up pepsin by receptor-mediated endocytosis. Moreover, exposure to pepsin (0.1 mg/mL) at neutral pH can induce mitochondrial and Golgi complex damage in hypopharyngeal epithelial cells. Cell damage may be caused by the transfer of pepsin to the Golgi complex, where it is activated at pH ~5.0 [30, 31]. Pepsin could be detected in nasal secretions and nasal turbinate mucosa in CRS patients [16], which proved that pepsin may cause damage to nasal tissues. In the current study, the cell death rate increased in HNEpCs after treatment with pepsin at pH 7.0, suggesting that nasal epithelial cell damage can be caused by non-acid reflux. The pH in the human nasal cavity ranges

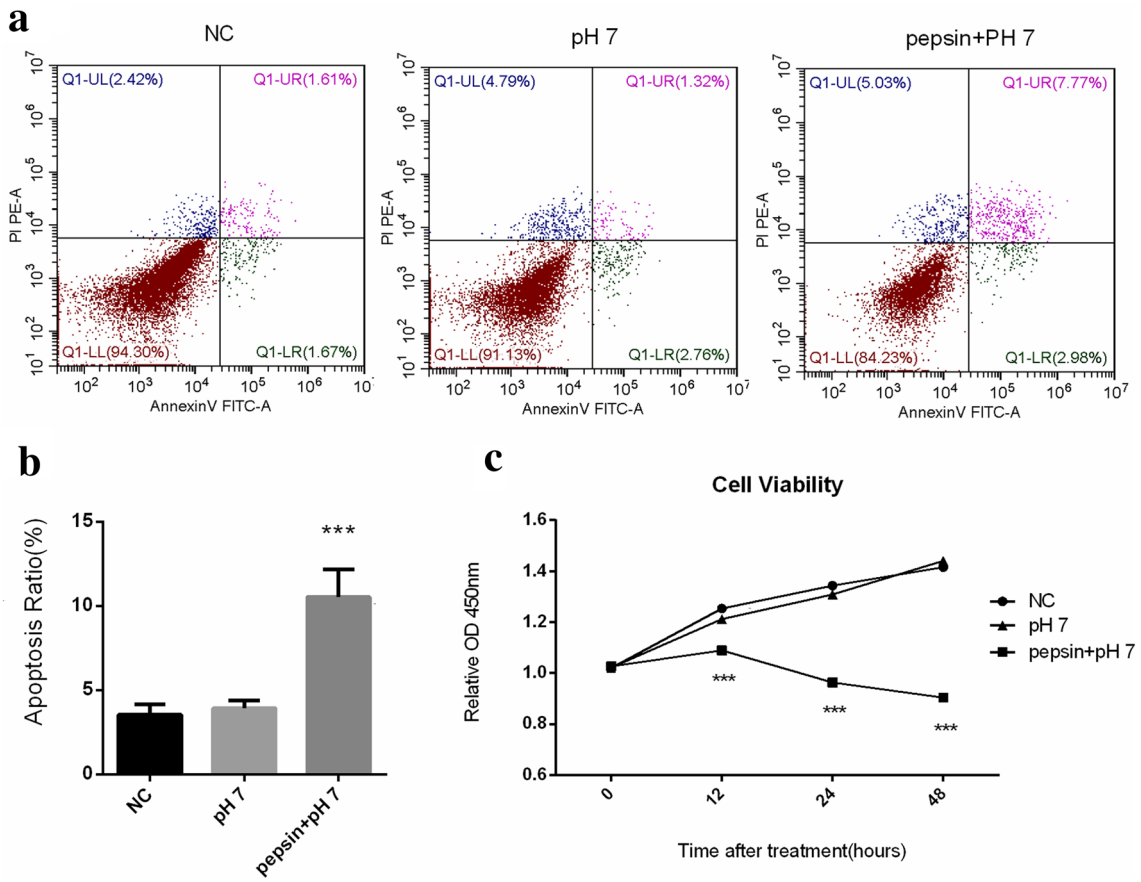

Fig. 5 Effect of pepsin on cell death in HNEpCs. **a, b** Flow cytometry assays and quantification of cell apoptosis in HNEpCs treated with pepsin at pH 7.0 or with pH 7.0 alone and in the control group using the annexin V-FITC apoptosis assay. **c** The Cell Counting Kit-8 assay indicated decreased cell viability in HNEpCs treated with pepsin at pH 7.0 or pH 7.0 alone and in the control group

between 5.2 and 8.1 [32]. Whether reflux events measured by nasopharyngeal pH monitoring are positively correlated with CRS is controversial [33]. However, until now, reflux events in the nasal cavity had not been measured.

HSP70, with a molecular weight of approximately 70 kDa, belongs to the HSP family and plays an important role in protective mechanisms. The expression of HSP70, a molecular chaperone, increases after acute or chronic stimulation in different human tissues [34, 35]. The previous studies have shown that HSPs protect myocardial cells from ischemia and enhance functional recovery [36, 37]. HSP70 also plays an important role in protecting human endothelial cells from apoptosis [38]. It was demonstrated in vitro that overexpression of HSP70 protects cells from cytotoxicity induced by a variety of cytokines [39–41]. In the current study, the expression of HSP70 was significantly higher in HNEpCs stimulated with pepsin than in control cells without pepsin. These results suggested that pepsin promoted the expression of HSP70, consistent with the previous studies in which HSP70 levels were significantly increased in pepsin-positive turbinate mucosa compared with pepsin-negative turbinate mucosa in CRS patients [16]. According to the previous studies, the expression of HSP70 in laryngeal tissue in LPR patients is increased. However, the relationship between pepsin and HSP70 expression has not been determined in vitro [15, 31]. One possible cause for this difference may be that the pathophysiological response to external stimuli differs among different human tissues. However, upregulation of HSP70 may not be sufficient to protect nasal epithelial cells against pepsin stimulation.

In the current study, SP-600125, a specific inhibitor of JNK1/2, decreased HSP70 protein expression in HNEpCs after pepsin stimulation. However, the other two inhibitors had no effect on HSP70 protein expression. These results indicate that JNK1/2 activation plays a role in the expression of HSP70 in response to pepsin in HNEpCs. Pepsin may increase the expression of HSP70 in HNEpCs via activation of the JNK/MAPK signaling pathway. The previous studies showed that acid reflux activates the MAPK signaling pathways in esophageal squamous cells of patients with gastroesophageal reflux disease, and the inhibition of p38 MAPK attenuates esophageal mucosal damage in vitro

[42, 43]. JNK/MAPK was demonstrated to be associated with cell survival and death under toxic stimuli [23]. The results of this study indicate that pepsin activates the JNK/MAPK signaling pathways in nasal epithelial cells. In addition, several studies have indicated that the MAPK signaling pathway regulates the expression of a variety of proteins, including HSP70, in response to different stimuli. Inhibitors of p38, ERK1/2, or JNK1/2 can prevent the upregulation of HSP70 protein expression in different cell types [26, 44, 45]. These results are consistent with those of the current study, which confirmed the vital role of the JNK/MAPK pathway in regulating HSP70 protein expression. This study showed the apoptosis in HNEpCs at 12 h after treatment with pepsin at pH 7.0 which was significantly increased compared with the control and pH 7.0 groups. Cell viability decreased following exposure to pepsin at pH 7.0. This further proved the damage on HNEpCs by pepsin stimulation under neutral pH. A limitation in the current study is the pathogenicity mechanism of CRS with reflux was not yet explored clearly. Further studies are needed to analyze the expression of other HSP family members after pepsin stimulation. Furthermore, activation of the MAPK signaling pathway should be evaluated in nasal tissues of CRS patients with LPR.

Conclusions

Pepsin, even under neutral pH, can increase the expression of HSP70 in HNEpCs by activating the JNK/MAPK signaling pathway. Increased HSP70 may be the protective mechanism when pepsin presents in the other parts of the body.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent None.

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