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The Expression and Regulation of Na^+-K^+-ATPase in Nasal Epithelial Cells of Chronic Rhinosinusitis with Nasal Polyps

Guangyi Ba[a, b, c] Ru Tang[a, b, c] Song Mao[a, b, c] Zhipeng Li[a, b, c] Haibo Ye[a, b, c] Hai Lin[a, b, c] Weitian Zhang[a, b, c]

[a]Department of Otolaryngology-Head and Neck Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, PR China; [b]Otolaryngological Institute, Shanghai Jiao Tong University, Shanghai, PR China; [c]Shanghai Key Laboratory of Sleep Disordered Breathing, Shanghai, PR China

Keywords
Chronic rhinosinusitis · Nasal polyp · Human nasal epithelial cell · Na^+-K^+-ATPase · Aquaporin-5 · Edema

Abstract
Objective: Na^+-K^+-ATPase (NKA) is essential in maintaining cell permeability, reserving potential energy, and preventing cellular edema. Nevertheless, how NKA expression is altered and regulated in chronic rhinosinusitis with nasal polyps (CRSwNPs) remain uncertain. Therefore, the present study aimed to explore the expression and regulation of NKA in CRSwNP. ***Methods:*** NKA immunolabeling was assessed by the immunohistochemistry method, NKA protein levels were detected with the Western blotting method, and mRNA levels of NKA and aquaporin-5 (AQP5) were assayed by real-time PCR in nasal tissues from CRSwNP and control subjects. The co-localization of NKA with inflammatory cells was evaluated by immunofluorescence staining. In addition, human nasal epithelial cells (HNECs) were cultured and stimulated using various stimulators to evaluate the regulation of NKA. ***Results:*** We found significantly decreased NKA positive cells, NKA protein levels, and mRNA levels of NKA and AQP5 in nasal tissues from CRSwNP patients compared to control subjects, especially in eosinophilic CRSwNP. Furthermore, NKA mRNA levels in HNECs were downregulated by staphylococcal enterotoxin B (SEB), lipopolysaccharides (LPSs), inflammatory cytokine (IFN)-γ, IL-4, IL-13, and IL-1β. ***Conclusion:*** NKA and AQP5 expressions were decreased in CRSwNP. NKA in HNECs could be suppressed by SEB, LPS, IFN-γ, IL-4, IL-13, and IL-1β. Impairment of NKA may contribute to the genesis and development of CRSwNP via inducing AQP5 downregulation and edema.

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNPs) is a multifactorial and heterogeneous disease with persistent and exaggerated inflammation in sinonasal mucosa, which negatively affects patient's quality of life and aggravates their financial burdens [1–3]. Considerable data indicate that CRSwNP is caused by the complicated interactions among various inflammatory cells and mediators [4–6], whereas, the precise pathogenetic mechanism of CRSwNP remains uncertain.

G. Ba and R. Tang contributed equally to this work.

karger@karger.com
www.karger.com/orl

Correspondence to:
Hai Lin, linh96@163.com
Weitian Zhang, drzhangwt@163.com

Na^+-K^+-ATPase ([NKA] also named as Na^+ pump or Na^+-K^+ pump) is an electrogenic transmembrane ATPase for exporting 3 Na^+ out of cells and importing 2 K^+ into cells against their electrochemical gradients simultaneously by consuming 1 ATP molecule to sustain the homeostasis of Na^+-K^+ [7]. NKA plays a crucial role in maintaining cell permeability, reserving potential energy, and preventing cellular edema [8]. Impairment of NKA could induce Na^+-K^+ homeostatic imbalance, leading to depolarization of the resting membrane potential, osmotic imbalance, and cellular edema [9, 10]. Previous reports indicate that impairment of NKA contributes to pulmonary edema and inflammation in acute respiratory distress syndrome [11, 12]. Aquaporin-5 (AQP5), one type of AQPs, has been showed to be essential in the liquid homeostasis of human nasal epithelial cells (HNECs). One previous report indicates that NKA and AQP5 levels were downregulated concurrently and then exacerbated the formation of edema by failed water clearance in the porcine reproductive and respiratory syndrome virus-infected pig lungs [14].

This study sought to explore the expression and regulation of NKA in CRSwNP. First, we assessed NKA immunolabeling and protein levels, mRNA levels of NKA and AQP5, and co-localization of NKA with inflammatory cells in nasal tissue samples from CRSwNP patients and control subjects. Second, in vitro studies, HNECs were cultured and stimulated using various stimulators to evaluate the regulation of NKA.

Materials and Methods

Subjects and Tissue Samples

A total of 88 subjects, consisting of 30 patients with eosinophilic CRSwNP (ECRSwNP), 32 patients with non-ECRSwNP (nECRSwNP), and 26 control subjects were enrolled in the present study. The diagnostic criteria of CRSwNP, ECRSwNP, and nECRSwNP were established according to previous published articles [15–17]. During endoscopic surgery, we collected NP, uncinate process (UP), and HNECs from CRSwNP patients and control subjects. Subjects without CRS undergoing skull base surgery, lacrimal duct surgery, or septoplasty for deviated septum were defined as control subjects whose UPs were collected as control tissues. Detailed characteristics of all the subjects are listed in online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000517101. This study was approved by the Ethical Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and we received written informed consents from all the study participants.

Immunohistochemistry (IHC) and Western blotting were employed to assess NKA immunolabeling and protein levels, respectively. In addition, real-time PCR was used to detect NKA and AQP5 mRNA levels in nasal tissue samples. The co-localization of NKA with inflammatory cells was evaluated by immunofluorescence staining. In vitro studies, HNECs were cultured and incubated with different stimulators, and then, real-time PCR and the NKA activity assay kit were performed to assess the effects and mechanism of stimulators on the cells. More detailed information is provided in the online suppl. material.

Histological, Immunohistochemical, and Immunofluorescent Staining

Histological, immunohistochemical, and immunofluorescent staining were performed as previously described [18]. Eosinophils were stained using hematoxylin-eosin staining and counted. IHC was employed to assess NKA expression in patients with CRSwNP and control subjects. Immunofluorescent staining was performed to evaluate the presence of NKA in CD3, CD20, CD138, CD68, CD163, major basic protein, myeloperoxidase, or tryptase positive cells.

Western Blotting

The expression of NKA protein in nasal tissues from patients with CRSwNP and control subjects was assayed with Western blotting methods.

Quantitative Real-Time Reverse Transcription PCR

Quantitative real-time PCR was employed to assess NKA and AQP5 mRNA expression in nasal tissues from patients with CRSwNP and control subjects. Total RNA in the collected tissues was extracted utilizing the RNeasy commercial kit (Qiagen, Chatsworth, CA, USA) as previously described [19, 20].

HNECs Culture and Stimulation

HNECs were scraped from 8 control subjects, 8 nECRSwNP patients, and 8 nECRSwNP patients, and cultured under air-liquid interface conditions as previously described [21]. Briefly, HNECs were incubated with different stimulators involving staphylococcal enterotoxin B (SEB), lipopolysaccharides (LPSs), poly I:C (dsRNA), cytokines including inflammatory cytokine (IFN)-γ, IL-4, IL-13, IL-17A, TNF-α, and IL-1β at a certain concentration (10 ng/mL, except SEB was 0.5 μg/mL, LPS was 1 μg/mL, and poly I:C was 25 μg/mL) for 24 h. Following stimulation, HNECs were collected for NKA mRNA and NKA activity measurement.

Quantitative Real-Time Reverse Transcription PCR

NKA mRNA levels of the incubated cells were assayed by the real-time PCR method.

NKA Activity Measurement

NKA activity of HNECs was assayed using the NKA activity assay kit according to the manufacturer's protocol.

Statistical Analysis

A p value <0.05 was deemed as statistically significant. Mann-Whitney U tests or Kruskal-Wallis H tests were applied to compare between-group or intergroup data of nasal tissue samples. The Spearman test was used to evaluate correlations. The paired t test was used to analyze cell culture data. Tissue sample data were expressed as dot plots with medians and interquartile ranges. Cell culture data were presented as mean ± standard error of the mean.

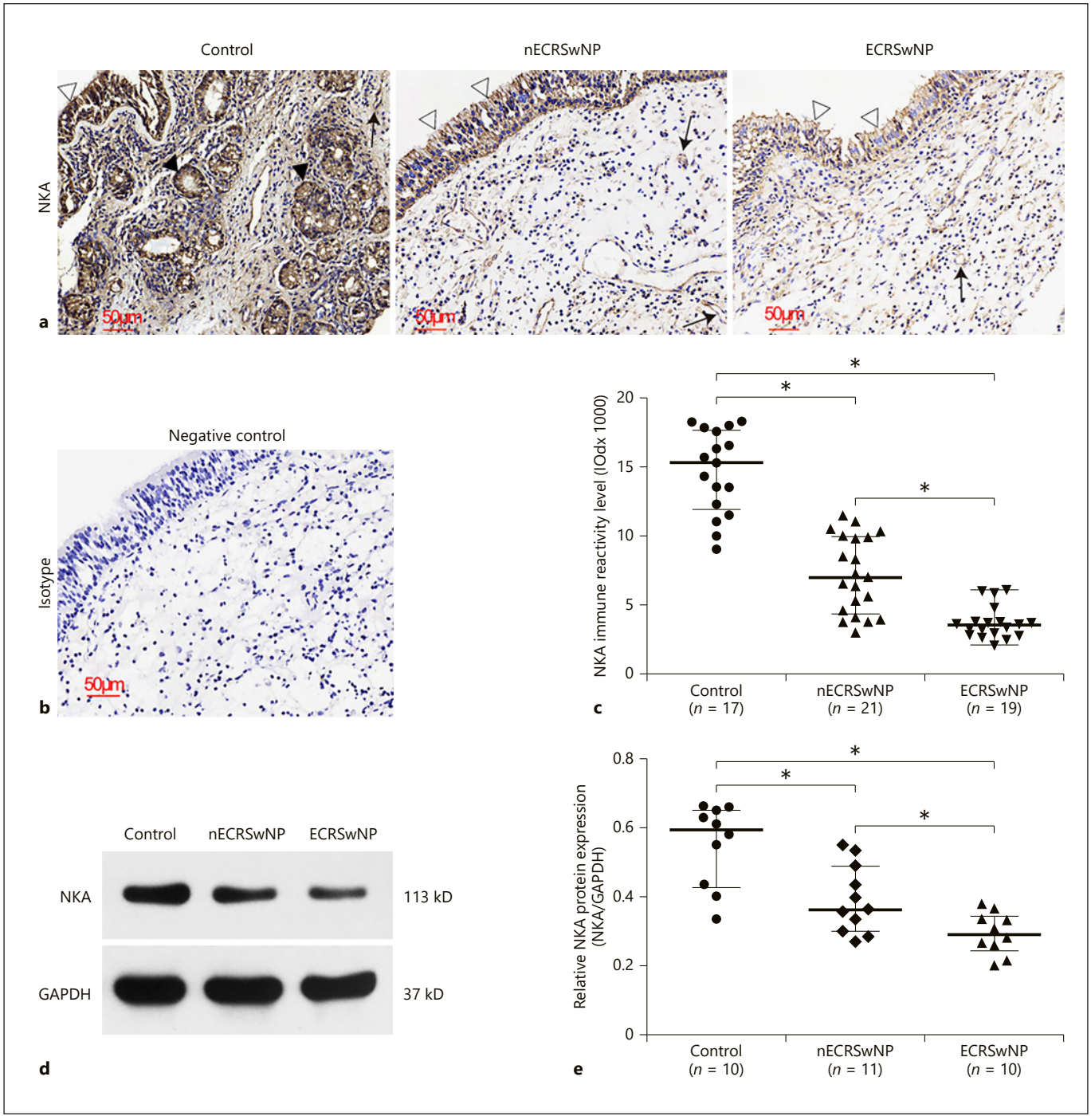

Fig. 1. IHC results of NKA positive cells in control tissues, nECRSwNP, and ECRSwNP (**a**). Negative control slide for IHC testing (**b**). The NKA immune reactivity level comparison among control tissues, nECRSwNP, and ECRSwNP (**c**). Western blotting results in control tissues, nECRSwNP, and ECRSwNP (**d**). Analysis of the relative NKA protein expression level in control tissues, nECRSwNP, and ECRSwNP (**e**). *$p < 0.05$. NKA, Na^+-K^+-ATPase; ECRSwNP, eosinophilic chronic rhinosinusitis with nasal polyp; nECRSwNP, noneosinophilic chronic rhinosinusitis with nasal polyp; IHC, immunohistochemistry.

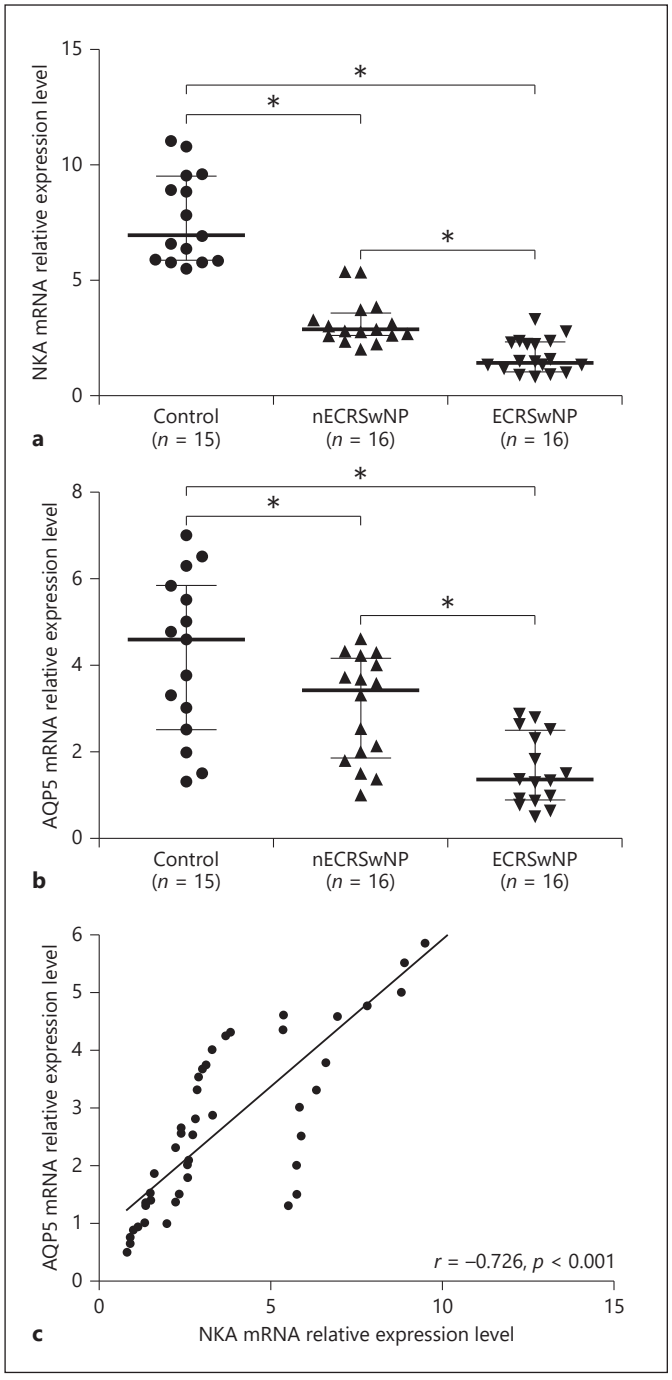

Fig. 2. Detection and analysis of NKA mRNA (**a**) and AQP5 mRNA (**b**) expression using real-time PCR in NPs and control tissues. Spearman analysis on the correlation of NKA and AQP5 mRNA levels (**c**). *$p < 0.05$. NKA, Na^+-K^+-ATPase; AQP5, aquaporin-5.

Results

Decreased NKA Immunolabeling in CRSwNP

NKAs were mainly distributed in the plasma membrane of nasal mucosal epithelial cells, glandular epithelial cells, and vascular endothelial cells (Fig. 1a). Decreased NKA positive cells were distributed in NP tissues from patients with CRSwNP including ECRSwNP and nECRSwNP compared to control UP samples (Fig. 1a–c). Furthermore, NKA immune reactivity levels were markedly downregulated in NP tissues from patients with ECRSwNP in contrast to that from patients with nECRSwNP (Fig. 1a–c).

Detection of NKA Protein Levels in Nasal Tissues

Of note, significantly weaker bands of NKA protein were detected in nasal tissue samples from CRSwNP patients compared to control UP samples, particularly in ECRSwNP (Fig. 1d, e).

Detection of NKA and AQP5 mRNA Levels in Nasal Tissues

Of interest, we also found lower mRNA levels of NKA and AQP5 in nasal tissues from patients with CRSwNP including ECRSwNP and nECRSwNP compared to UP samples from control subjects, particularly in ECRSwNP (Fig. 2a, b). In addition, NKA mNRA levels were positively correlated with AQP5 mRNA levels in samples from CRSwNP and control subjects (Spearman's test, $r = 0.726$, $p < 0.001$; Fig. 2c).

Co-Localization of NKA in Inflammatory Cells

Dual immunofluorescent staining was used to evaluate co-localization of NKA with inflammatory cells including CD3 (T cell), CD20 (B cell), CD138 (plasma cell), CD68 (pan macrophage), CD163 (M2 macrophage), major basic protein (eosinophil), myeloperoxidase (neutrophil), and tryptase (mast cell). Notably, NKA was expressed weakly in a minority of mast cells and plasma cells (Fig. 3a, b), and NKA was not detected in pan macrophages, M2 macrophages, eosinophils, neutrophils, T cells, and B cells (Fig. 3c–h).

Effects of Various Stimulators on NKA mRNA Levels and NKA Activities in HNECs

In vitro studies, HNECs were cultured and stimulated using various stimulators to study the regulation of NKA in HNECs. Interestingly, we found significant downregulation of NKA mRNA levels and NKA activities in the HNECs following SEB, LPS, IFN-γ, IL-4, IL-13, and IL-1β treatment, respectively (Fig. 4a, b). In addition, no significant changes in NKA mRNA levels and NKA activities were observed after poly I:C, IL-17A, and TNF-α (Fig. 4a, b).

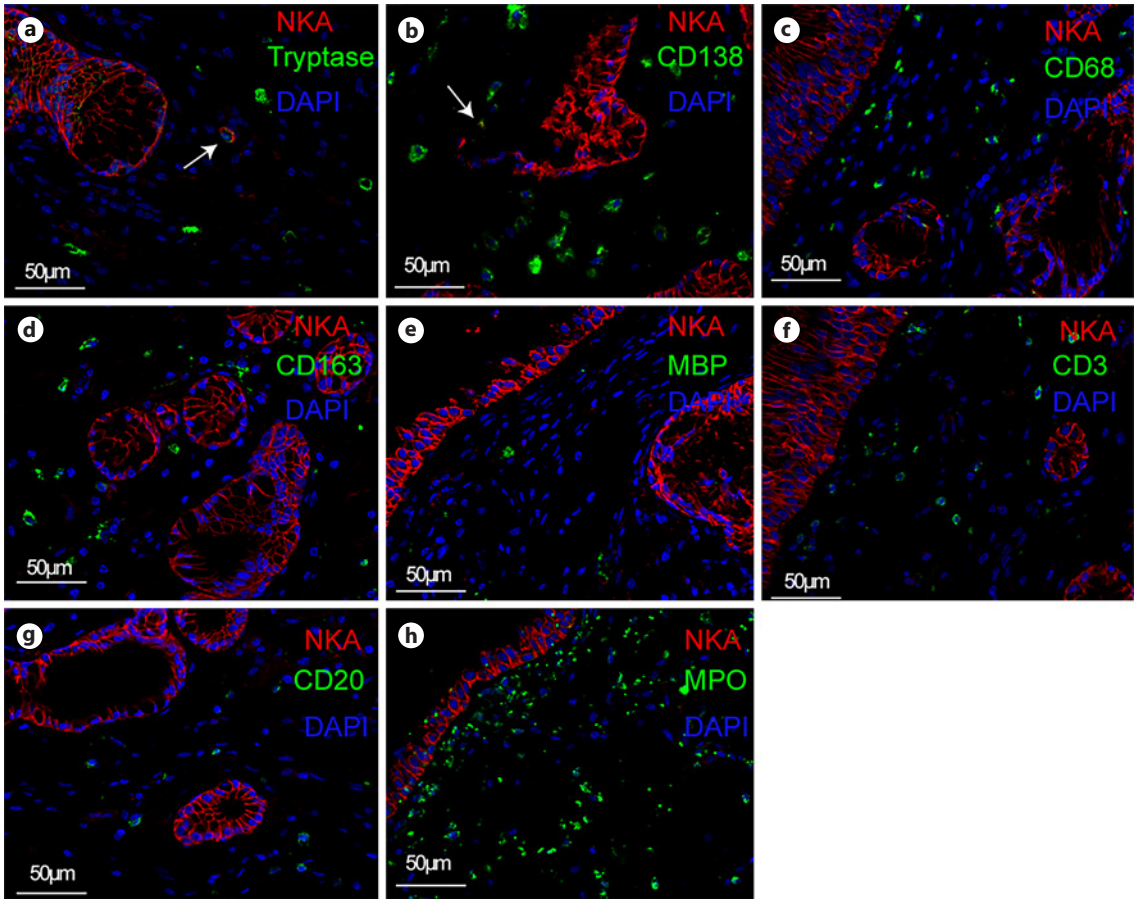

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Fig. 3. Immunofluorescent staining showed co-localization of NKA with tryptase (mast cell, **a**), CD138 (plasma cell, **b**), CD68 (pan macrophage, **c**), CD163 (M2 macrophage, **d**), and MBP (eosinophil, **e**) in NP tissues. Immunofluorescent staining showed no co-localization of NKA with CD3 (T cell, **f**), CD20 (B cell, **g**) and MPO (neutrophil, **h**) in NP tissues. Scale bar, 50 μm. Arrow indicates co-localization of NKA with cell marker. NKA, Na^+-K^+-ATPase; MBP, major basic protein; MPO, myeloperoxidase.

Discussion

Previous studies have indicated that numerous glands and few edema in sinonasal mucosa are histopathological characteristics of normal nasal tissues, while few glands, obvious edema, and exacerbated inflammation in sinonasal mucosa are main histopathological characteristics of CRSwNP [22], whereas, the etiology and mechanism of the characteristics of CRSwNP remain unclear. There is ample evidence that homeostasis of Na^+ and K^+ is essential in cell life activities, and NKA serves to regulate this homeostasis by exporting Na^+ and importing K+ in a variety of cells [9]. NKA dysfunction can lead to Na^+-K^+ homeostatic imbalance, osmotic imbalance, and cellular edema [23]. However, whether NKA expression is altered and regulated in CRSwNP remains unknown. Therefore, this study aimed to explore the expression and regulation of NKA in CRSwNP.

As indicated in IHC findings, NKA was mainly distributed in the plasma membrane of nasal mucosal epithelial cells, glandular epithelial cells, and vascular endothelial cells, and decreased NKA levels were found in CRSwNP compared to control UP samples, particularly in ECRSwNP, indicating that NKA function is impaired in nasal mucosal epithelial cells, glandular epithelial cells, and vascular endothelial cells in CRSwNP, particularly in ECRSwNP. Using IHC, Western blotting, and real-time PCR methods, we demonstrated that NKA positive cells and protein levels, and mRNA levels of NKA and AQP5 were decreased in nasal tissues from CRSwNP patients, especially ECRSwNP patients, indicating that NKA and AQP5 levels are downregulated in CRSwNP. As a corollary, impairment of NKA may

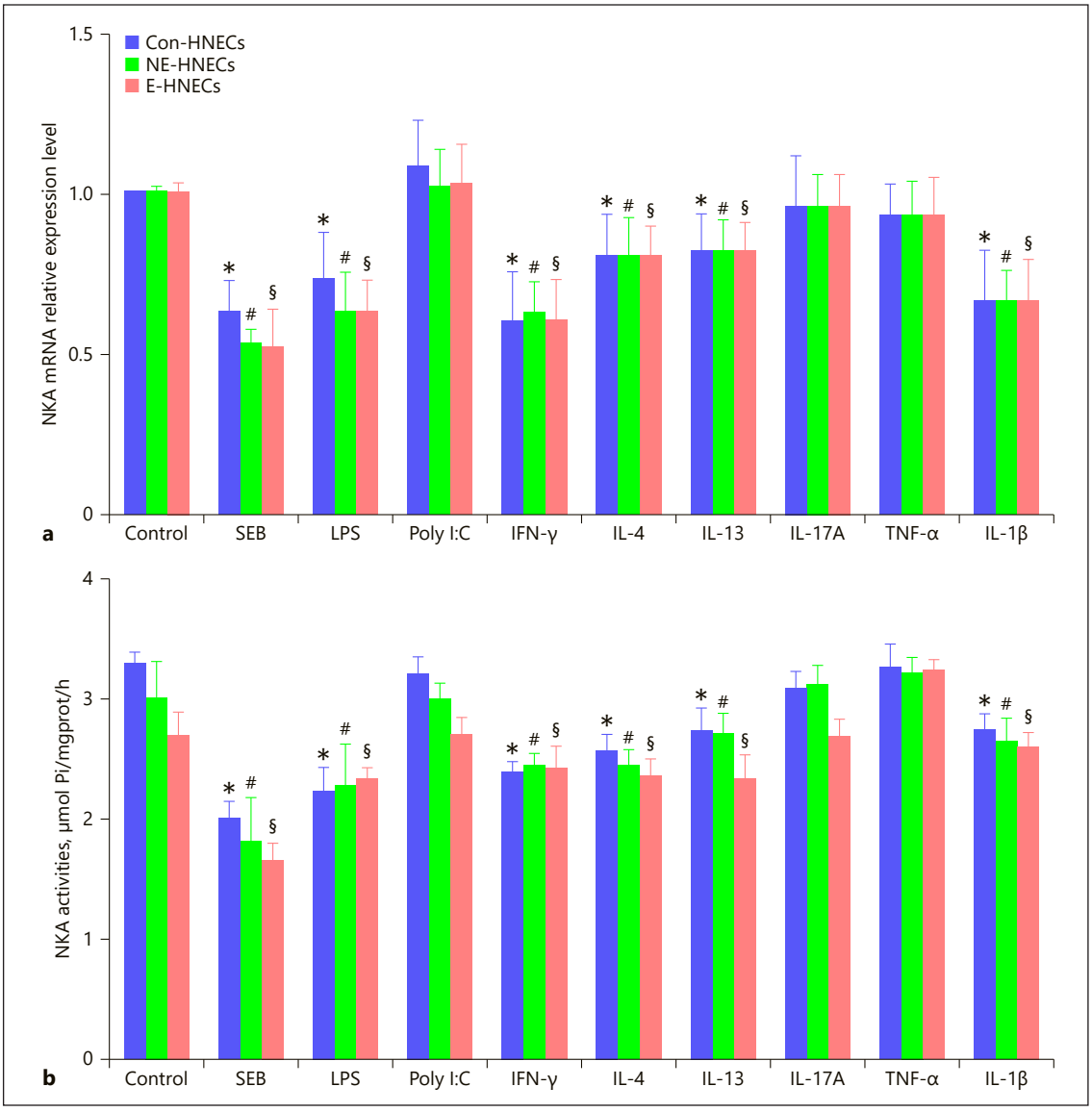

Fig. 4. Effects of various stimulators on NKA mRNA levels (**a**) and NKA activities (**b**) in Con-HNECs, NE-HENCs, and E-HNECs. Con-HNECs indicate HNECs from control subjects, NE-HENCs indicate HNECs from nECRSwNP patients, and E-HNECs indicate HNECs from ECRSwNP patients. $^{*}p < 0.05$ versus Con-HNECs, $^{\#}p < 0.05$ versus NE-HENCs and $^{\S}p < 0.05$ versus NE-HENCs in the control groups without any treatment. NKA, Na^{+}-K^{+}-ATPase; HNECs, human nasal epithelial cells; ECRSwNP, eosinophilic chronic rhinosinusitis with nasal polyp; nECRSwNP, noneosinophilic chronic rhinosinusitis with nasal polyp.

lead to AQP5 downregulation, resulting in the impairment of maintaining cell permeability, reserving potential energy, and preventing cellular edema, which may be related with edema formation in CRSwNP, particularly in ECRSwNP which has more exacerbated and persistent inflammation compared to nECRSwNP. Previous studies have shown that inverse relationship between NKA activity and inflammation degree was involved in a variety of inflammatory diseases [24, 25]. As a corollary, the lower NKA levels in CRSwNP, the more severe the inflammation in CRSwNP, indicating that NKA may be used as an important indicator to predict the degree of inflammation in CRSwNP, which would provide guidances for clinical diagnosis of CRSwNP and distinguishing between ECRSwNP and nECRSwNP. In our opinion, both reasons including fewer total cells and the level of NKA expression are reduced in each cell in ECRSwNP compared to nECRSwNP or control UPs may be related to NKA downregulation in ECRSwNP, in other words, exacer-

bated inflammation in ECRSwNP could cause fewer NKA-expressing cells and lower NKA levels in each cell.

Among inflammatory cells, NKA was expressed weakly in a minority of mast cells and plasma cells and not detected in pan macrophages, M2 macrophages, eosinophils, neutrophils, T cells, and B cells. These results indicate that NKA function may be impaired in mast cells and plasma cells, pan macrophages, M2 macrophages, eosinophils, neutrophils, T cells, and B cells. Less NKA localization to these inflammatory cells may suggest that NKA may not exert its function on these inflammatory cells just like abovementioned epithelial cells, and a novel treatment targeting NKA should be performed on epithelial cells instead of these inflammatory cells, which would provide guidances for clinical targeted NKA therapy on CRSwNP in the future.

In HNECs culture and stimulation experiment, NKA mRNA levels and NKA activities were significantly suppressed by SEB, LPS, IFN-γ, IL-4, IL-13, and IL-1β. These findings were partially in line with previous reports indicating that NKA expression or activity could be suppressed by SEB, LPS, IFN-γ, IL-1β, or TNF-α intervention on a variety of tissues and cells including rat intestinal mucosa [26], rat astrocytes [27], human intestinal xenografts [28], rat cardiac myocytes [29], and HepG2 cells (a human liver cancer cell line) [30], et al. Previous studies have indicated the SEB and LPS could exacerbate inflammatory responses of HNECs in CRSwNP [31–33], thus, collectively, it can be surmised that NKA function in HNECs is impaired following SEB and LPS treatment, indicating that bacterial infection could trigger aggravation of inflammation via impairing NKA function in HNECs. In addition, type 1 IFN-γ, type 2 IFNs (IL-4 and IL-13), and IL-1β could also exert important roles in the inflammatory responses in CRSwNP [4, 34, 35]; therefore, it is reasonable to speculate that NKA function in HNECs is also impaired following type 1 IFN-γ, type 2 IFNs (IL-4 and IL-13), and IL-1β stimulation, indicating that these IFNs could also induce trigger inflammatory responses via impairing NKA function in HNECs.

In addition, no significant changes in NKA mRNA levels and NKA activities were observed after poly I:C, IL-17A, and TNF-α stimulation. To some extent, this discrepancy may be due to the differences between HNECs and other tissues and cells.

Some limitations of our study should be noted. First, due to the difficulty of isolating glandular epithelial cells and vascular endothelial cells from nasal tissues, we only used nasal epithelial cells to perform in vitro studies, glandular epithelial cells and vascular endothelial cells need to be isolated to further precisely assess the effects and mechanism of NKA in CRSwNP in the future. Second, we only study NKA expression and regulation in CRSwNP, downstream signaling pathway of NKA in CRSwNP needs to be further explored and clarified in the future to make a more detailed and precise evaluation on the role and mechanism of NKA in CRSwNP.

Conclusion

NKA and AQP5 expression were decreased in CRSwNP. NKA in HNECs could be suppressed by SEB, LPS, IFN-γ, IL-4, IL-13, and IL-1β. Impairment of NKA may contribute to the genesis and development of CRSwNP via inducing AQP5 downregulation and edema.

Statement of Ethics

This study was approved by the Ethical Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (no.: 2019-KY-039[K]), and we received written informed consents from all the study participants.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

Hai Lin and Weitian Zhang conceived and designed the experiments. Guangyi Ba and Ru Tang performed the experiments. Hai Lin, Guangyi Ba, Ru Tang, Zhipeng Li, Song Mao, and Haibo Ye collected specimens and analyzed the data. Guangyi Ba, Hai Lin, and Weitian Zhang wrote and revised the article.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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