

# Hypoxia Increases Epithelial Permeability in Human Nasal Epithelia

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**Purpose:** The nasal mucosa is the first site to encounter pathogens, and it forms continuous barriers to various stimuli. This barrier function is very important in the innate defense mechanism. Additionally, inflammation of the nasal sinus is known to be a hypoxic condition. Here, we studied the effect of hypoxia on barrier function in normal human nasal epithelial (NHNE) cells. **Materials and Methods:** The expression levels of various junction complex proteins were assessed in hypoxia-stimulated NHNE cells and human nasal mucosal tissues. We performed real-time polymerase chain reaction analysis, western blotting, and immunofluorescence assays to examine differences in the mRNA and protein expression of ZO-1, a tight junction protein, and E-cadherin in NHNE cells. Moreover, we evaluated the trans-epithelial resistance (TER) of NHNE cells under hypoxic conditions to check for changes in permeability. The expression of ZO-1 and E-cadherin was measured in human nasal mucosa samples by western blotting. **Results:** Hypoxia time-dependently decreased the expression of ZO-1 and E-cadherin at the gene and protein levels. In addition, hypoxia decreased the TER of NHNE cells, which indicates increased permeability. Human nasal mucosa samples, which are supposed to be hypoxic, showed significantly decreased levels of ZO-1 and E-cadherin expression compared with control. **Conclusion:** Our results demonstrate that hypoxia altered the expression of junction complex molecules and increased epithelial permeability in human nasal epithelia. This suggests that hypoxia causes barrier dysfunction. Furthermore, it may be associated with innate immune dysfunction after encountering pathogens.

**Key Words:** Hypoxia, ZO-1, E-cadherin, permeability

## INTRODUCTION

The nasal mucosa is the first site of exposure to various inhaled pathogens, therefore, it is important in the innate defense mechanism. The epithelia of the nasal mucosa form continuous barriers and separate the external environment from the upper airway.<sup>1,2</sup> The physical barrier of epithelial cells is composed of tight and adherens junctions. Tight junctions are the most apical structure of the apical com-

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plex and disconnect the apical space from the basolateral area.<sup>3</sup> In addition, tight junctions maintain polarity by forming the fence-like barrier and controlling the flow of water, including solutes, through the paracellular space.<sup>4,5</sup> ZO-1 is a tight junction component that is observed in the uppermost layers of the nasal mucosal epithelium, and it colocalizes with other tight junction proteins.<sup>6</sup> Adherens junctions are crucial for the initiation and maintenance of intercellular adhesion, and they function as a docking site for signaling molecules.<sup>7,8</sup> E-cadherin is a transmembrane protein that forms calcium-dependent homophilic intercellular adhesions between epithelial cells,<sup>9</sup> and it is the main component of adherens junctions. The alteration of ZO-1 and E-cadherin expression has been shown in human nasal epithelial cells under many pathological conditions.<sup>10</sup>

Chronic sinusitis comprises several distinct disorders, including those characterized by anatomical obstruction to the sinus ostia with chronic/recurrent infection and remodeling.<sup>11</sup> Obstruction of the natural ostium of the sinus can induce mucosal hypoxia. Mucosal edema or polyps can also occlude natural ostium of the sinus, and inflammatory mucosa caused by chronic inflammation can induce hypoxia, even in a ventilated sinus.<sup>11,12</sup> One study revealed that the oxygen tension is significantly lower in inflamed sinuses than in non-inflamed sinuses, and the transient obstruction of the middle meatus results in the appearance of pathological radiographic findings.<sup>13</sup> Hypoxia activates the hypoxia-inducible factor (HIF)-1 $\alpha$  and 2 $\alpha$  transcription complex, which leads to the formation of new vessels.<sup>14</sup> Hypoxia-exposed nasal polyp-derived fibroblasts release CXCL8 (interleukin-8) and C-C motif chemokine,<sup>11</sup> which are involved in inflammatory cell recruitment.<sup>12</sup> Treatment of nasal fibroblasts with hypoxia increases the expression of vascular endothelial growth factor,<sup>12</sup> which mediates angiogenesis and increases vascular permeability.<sup>15</sup> Furthermore, inhibition of HIF-1 $\alpha$  reduces nasal inflammation in a murine allergic rhinitis model<sup>16</sup> and attenuates antigen-induced airway inflammation and hyper-responsiveness.<sup>17,18</sup> Therefore, the role of hypoxia in upper airway inflammation is attracting more attention from ear, nose, and throat specialists. Despite the importance of hypoxic conditions in the pathogenesis of sinusitis, no studies on the effects of hypoxia on barrier function in human nasal epithelial cells have been reported.

In this study, we hypothesized that hypoxic conditions may influence the physical barrier function of human nasal epithelial cells. To test this hypothesis, we investigated the effects of hypoxia on ZO-1 and E-cadherin expression and

measured the trans-epithelial resistance (TER) of cells.

## MATERIALS AND METHODS

### Normal human nasal epithelial (NHNE) cell culture and hypoxic conditions

After the approval of the study protocol by the Institutional Review Board of the Yonsei University College of Medicine (4-2012-0317), human nasal inferior turbinate mucosa samples were collected, and NHNE cells were harvested from chronic sinusitis patients who underwent endoscopic sinus surgery. NHNE cell culture was performed as previously described.<sup>19</sup> Briefly, cells (passage 2) were seeded in culture medium containing a 1:1 mixture of basal epithelial growth medium: Dulbecco's Modified Eagle Medium with all the supplements as previously described.<sup>20</sup> Culture media (0.5 mL) containing NHNE cells were placed onto a 24.5-mm, 0.45-mm-pore Transwell-clear (Costar Co., Cambridge, MA, USA) culture insert. Cells were maintained until they were submerged for the first 9 days, and the culture medium was changed on Day 1 and every other day thereafter. The air-liquid interface (ALI) was formed by removing the apical culture medium on Day 9. The culture medium on the basal area of the culture plate was replaced daily, and NHNE cells on ALI Day 14 were used in all experiments. All experiments were repeated three times using different batches of NHNE cells. On ALI Day 14, hypoxia was induced by placing cells in a humidified hypoxic incubator (1% O<sub>2</sub>, 5% CO<sub>2</sub>, 37°C).

### Real-time polymerase chain reaction (PCR)

Following hypoxia treatment, NHNE cells were harvested for total RNA extraction. The cells were homogenized in Trizol Reagent (Sigma-Aldrich, St. Louis, MO, USA), and real-time PCR was performed according to the manufacturer's protocol. The primers ( $\beta$ -actin fwd: 5' GCCAACCGC GAGAAGATG-3', rev: 5' ACGGCCAGAGGCGTACAG-3'; ZO-1 fwd: 5' -TGGTGTCTACCTAAT TCAACTCA-3', rev: 5' -CGCCAGCTACAAATATTC CAACA-3'; E-cadherin fwd: 5' -ATAGAGAACGCATT GCCACATACA-3', and rev: 5' -TTCTGATCGGTTACC GTGATCA-3') were synthesized, and quantitative reverse transcription-PCR was performed using the TaqMan<sup>®</sup> universal PCR master mix (Applied biosystems, Foster City, CA, USA). Target-specific probes were purchased from Applied Biosystems. All reactions were performed in tripli-

cate using different batches of NHNE cells. The relative mRNA quantities were determined using the comparative threshold method, and the results were normalized against  $\beta$ -actin.

### Western blotting

Western blotting was performed to assess the changes in the protein expression levels of ZO-1 and E-cadherin. Thus, NHNE cells were homogenized in radioimmunoprecipitation assay lysis buffer (Thermo, Rockford, IL, USA) containing a mixture of protease inhibitors (Sigma-Aldrich). The bicinchoninic acid (Thermo) assay was used to quantify the amount of extracted protein. Protein samples (40  $\mu$ g) were loaded on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Primary antibodies against ZO-1 (Invitrogen), E-cadherin (Cell Signaling), and  $\beta$ -actin (SantaCruz Biotechnology, Santa Cruz, CA, USA) were used. The secondary antibody of horseradish peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from Jackson Labs. Relative band intensities were measured using the ImageJ program (NIH). All experiments were repeated three times using different batches of NHNE cells.

### Immunofluorescence analysis

The changes in the expressions of ZO-1 and E-cadherin were analyzed by immunofluorescence analysis. NHNE cells were fixed with 4% paraformaldehyde for 20 min at room temperature (RT) and then treated with 0.1% Triton X-100 for 5 min. Blocking was performed for 30 min at RT with 3% bovine serum albumin in phosphate-buffered saline. Cells were incubated with rabbit anti-human ZO-1 (Invitrogen) or rabbit anti-human E-cadherin (Cell Signaling) antibodies overnight at 4°C and then incubated with Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen) for 1 hour at RT. Afterwards, the nucleus was stained with DAPI solution (1:1000, Invitrogen) for 1 minute at RT. Between each step, the cells were extensively rinsed three times for 5 minutes each time. Images were taken with a confocal microscope (Olympus FV-1000, Olympus, Tokyo, Japan). All experiments were repeated three times using different batches of NHNE cells.

### TER measurements

The effect of hypoxia on the disruption of barrier function was assessed by measuring the TER of NHNE cells. The TER between epithelial cells was measured using the

EVOM™ Epithelial Voltmeter. NHNE cells were incubated under hypoxic conditions for 8 hours, and the ENDOHM-12 electrode was placed immediately into both apical and basal culture media. The results are expressed as standard units of ohms per square centimeter ( $\Omega/\text{cm}^2$ ) by calculating the differences between values that were detected in the apical and basal culture media. We repeated the experiments four times with different batches of NHNE cells.

### Human samples

The study protocol was approved by the Institutional Review Board of the Yonsei University College of Medicine (4-2012-0317). Human nasal mucosa samples were harvested from ten subjects who were diagnosed with bilateral chronic sinusitis and underwent endoscopic sinus surgery. Chronic sinusitis was diagnosed based on preoperative computer tomography (CT) scans, physical examinations, and subjective symptoms, as assessed by a nose specialist and based on previously reported guidelines.<sup>21</sup> We harvested sinus mucosa samples from the maxillary sinus. The mucosa of the totally obstructed sinus, which is supposed to be more hypoxic, was compared with partially obstructed sinus mucosa samples from the same patients. The obstruction of the sinus ostium was evaluated based on the preoperative CT scan. The sinus mucosa was harvested immediately after the opening of the natural ostium, and it was stored at -70°C.

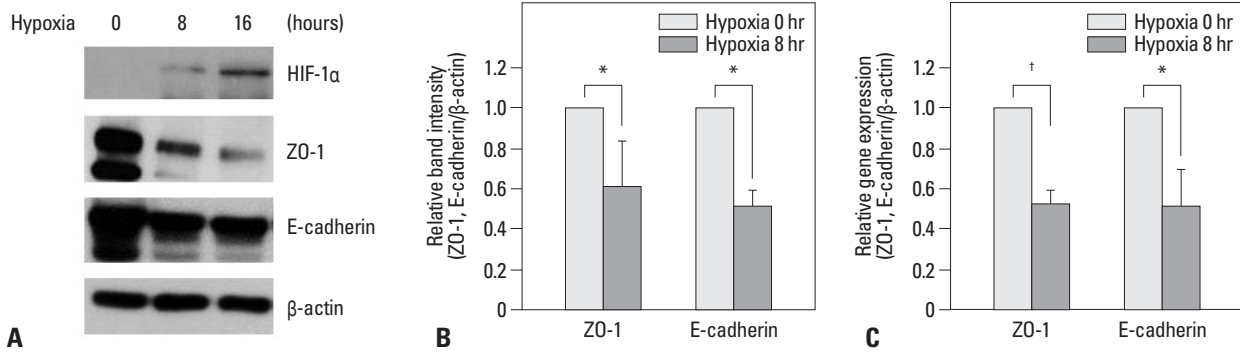
### Statistics

Data are presented as mean  $\pm$  standard deviation, which were calculated from at least three independent experiments. Statistical analysis was performed with the t-test using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).  $p < 0.05$  was considered to be statistically significant.

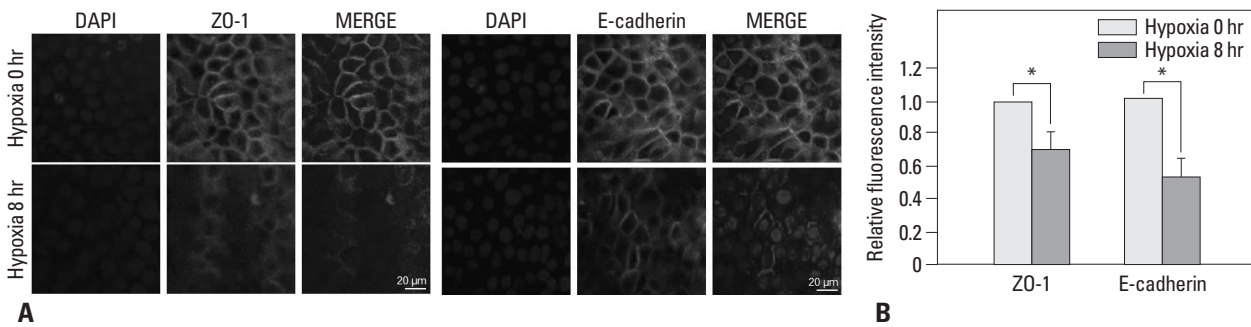
## RESULTS

### Hypoxia decreases the expression of ZO-1 and E-cadherin in NHNE cells

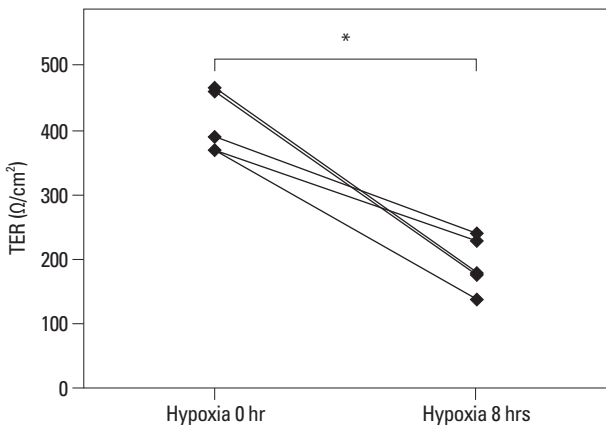
Exposure to hypoxia induced the protein expression of HIF-1 $\alpha$  in NHNE cells (Fig. 1A). Next, the protein expression levels of ZO-1 and E-cadherin were assessed by western blotting in NHNE cells that were incubated under hypoxic conditions for 0, 8, or 16 hours. Hypoxia decreased the expression of ZO-1 in a time-dependent manner. The expression of E-cadherin was decreased after 8 hours of hypoxia, and its level did not change until the 16-hour time



**Fig. 1.** Hypoxia decreases the levels of ZO-1 and E-cadherin in NHNE cells. (A) NHNE cells were incubated under hypoxic conditions, and Western blotting was performed to assess the expressions of HIF-1α, ZO-1, and E-cadherin at 8 and 16 hours of hypoxia. (B) The relative band intensities of ZO-1 and E-cadherin after 8 hours of hypoxia were calculated and normalized to β-actin. (C) NHNE cells were incubated under hypoxic conditions for 8 hours, and real-time PCR analysis was performed to compare the mRNA expressions of ZO-1 and E-cadherin. The relative gene expression was calculated by normalization to β-actin (n=3, \*p<0.05, †p<0.01). HIF, hypoxia-inducible factor; NHNE, normal human nasal epithelial; PCR, polymerase chain reaction.



**Fig. 2.** Immunofluorescence analysis of NHNE cells. (A) NHNE cells were incubated under hypoxic conditions for 8 hours, and the immunofluorescence assay was performed with ZO-1 and E-cadherin antibodies. Fluorescence images were taken using a confocal microscope. (B) The mean fluorescence intensity was calculated using the ImageJ program (n=3, \*p<0.05). NHNE, normal human nasal epithelial.



**Fig. 3.** Hypoxia decreases the TER of NHNE cells. NHNE cells were incubated under hypoxic conditions for 8 hours, and TER values were measured immediately. The mean TER values of control and hypoxia-conditioned cells were 407.5±43.76 and 199.5±41.17, respectively (n=4, \*p<0.01). TER, trans-epithelial resistance; NHNE, normal human nasal epithelial.

point (Fig. 1A). We set the duration of hypoxia treatment at 8 hours because the protein levels of ZO-1 and E-cadherin significantly decreased after 8 hours of hypoxia (Fig. 1B). These experiments were performed in triplicate. In addition, the mRNA expression levels of ZO-1 and E-cadherin were significantly reduced at 8 hours after hypoxia expo-

sure (Fig. 1C).

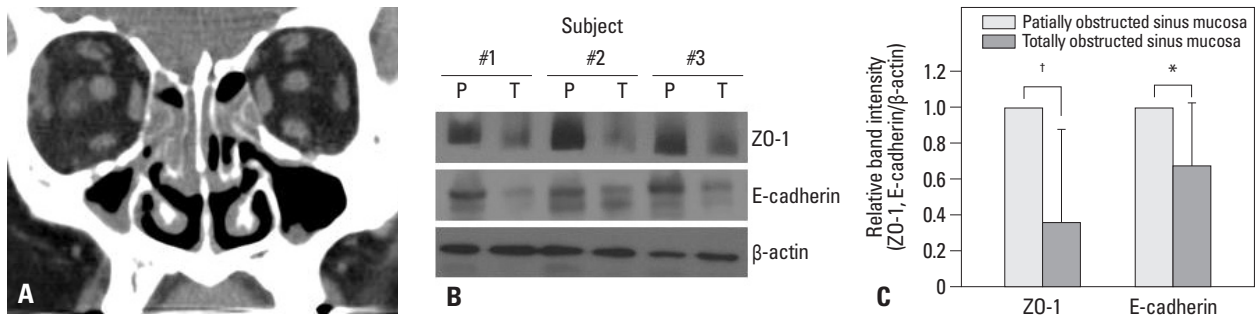
Next, we performed immunofluorescence staining to assess the expression patterns of ZO-1 and E-cadherin after 8 hours of hypoxia. Both ZO-1 and E-cadherin (green fluorescence) were observed at the borders between cells. The fluorescence intensities of ZO-1 and E-cadherin were significantly decreased after 8 hours of hypoxia, and these experiments were repeated 3 times (Fig. 2).

### Hypoxia decreases the TER of NHNE cells

Next, we compared the TER of NHNE cells, which were incubated under normal or hypoxic conditions for 8 hours. The experiments were repeated four times in different batches of cells. The mean values of TER were 407.5±43.76 and 199.5±41.17 in control and hypoxia-conditioned NHNE cells, respectively, and the decrease in TER was statistically significant (Fig. 3).

### The expressions of ZO-1 and E-cadherin are decreased in the hypoxia-conditioned human nasal mucosa

The mucosa of partially or totally obstructed maxillary sinus was harvested from patients who underwent bilateral endo-



**Fig. 4.** ZO-1 and E-cadherin expressions are decreased in the hypoxia-conditioned human nasal mucosa. (A) The maxillary sinus mucosa was harvested from totally (left) and partially (right) obstructed maxillary sinuses, and Western blotting was performed to compare the expressions of ZO-1 and E-cadherin. (B) The expressions of ZO-1 and E-cadherin were decreased in the totally obstructed maxillary sinus mucosa compared with the partially obstructed maxillary sinus mucosa. (C) The experiments were repeated in ten patients, and the relative band intensities of ZO-1 and E-cadherin were calculated and normalized to β-actin. P, sinus mucosa from the partially obstructed maxillary sinus; T, sinus mucosa from the totally obstructed maxillary sinus (n=10, \* $p$ <0.05, † $p$ <0.01).

scopic sinus surgery, and western blotting was performed to compare the expression levels of junction complex proteins. The expressions of ZO-1 and E-cadherin were significantly reduced in the sinus mucosa of totally obstructed maxillary sinuses compared with those in partially obstructed maxillary sinuses. We repeated the same experiments in ten patients with chronic rhino-sinusitis (Fig. 4).

## DISCUSSION

Many pathological conditions bring about alterations in the expression of tight and adherens junction proteins in various organs. In the colon, treatment with *Clostridium perfringens* decreases the protein level of E-cadherin.<sup>22</sup> In a mouse model, the Japanese encephalitis virus infection damages the integrity of the blood-brain barrier, demonstrated by reductions in ZO-1, occludin, and claudins.<sup>23</sup> In the human nasal mucosa, the virus infection causes the loss of ZO-1 from tight junction complexes, and the loss leads to intranasal bacterial inoculation in mice.<sup>24</sup> In our study, the mRNA and protein expression levels of ZO-1 and E-cadherin were decreased in hypoxia-stimulated NHNE cells. This is the first study to reveal a relationship between hypoxia and barrier dysfunction in upper airway epithelial cells by analyzing the expressions of ZO-1 and E-cadherin in the normal and hypoxic human nasal mucosa. Similar to our results, barrier function in the corneal epithelium has been shown to be disrupted by hypoxia, evidenced by down-regulation of ZO-1 expression.<sup>25</sup>

Unexpectedly, we found that the time courses of ZO-1 and E-cadherin regulation were somewhat different. Although hypoxia time-dependently decreased the protein expression of ZO-1 until the 16-hour time point, the protein

expression of E-cadherin at the 16-hour time point was not lower than that at the 8-hour time point. This suggests that ZO-1 and E-cadherin may be regulated independently. Similarly, when NHNE cells were re-oxygenated after hypoxic stimulation, Western blot assay indicated that the protein expression of E-cadherin was recovered time dependently, however, the recovery of ZO-1 expression was not definite by (Supplementary Fig. 1, only online). The TER value was also recovered after 24 hours of re-oxygenation (Supplementary Fig. 1, only online).

Several studies have reported changes in tight junction- and adherens junction-associated proteins in nasal pathological conditions. The mRNA level of ZO-1 is reduced in the allergic nasal mucosa.<sup>26</sup> In nasal polyp epithelia, the expression of ZO-1 is down-regulated, whereas E-cadherin is up-regulated.<sup>27</sup> Rhinovirus infection decreases the expression of tight and adherens junction components, which leads to the reduction of the TER of primary human nasal epithelial cells.<sup>10</sup> However, the exact mechanism involved in the alteration of tight and adherens junction components has not been shown in nasal epithelial cells. In the present study, we found that hypoxia increased the expression of HIF1- $\alpha$  time-dependently until 16 hours in NHNE cells. Further investigation is required to elucidate the inter-signaling molecule between ZO-1/E-cadherin and hypoxia in the upper airway epithelium.

TER has been shown to correlate with the number of tight junctions, and changes in TER values represent functional changes in paracellular permeability.<sup>10,28</sup> Therefore, the reduction in TER in our study implies an increase in paracellular permeability and suggests epithelial barrier dysfunction. Hypoxia has been shown to play various roles in the pathogenesis of rhino-sinusitis. It induces epithelial-to-mesenchymal transition and contributes to nasal polyposis. It



also releases chemokines in nasal epithelial cells, thus leading to the migration of eosinophils and neutrophils.<sup>29</sup> Furthermore, significantly lower oxygen tension has been found in the inflammatory sinus than in the non-inflammatory sinus.<sup>13</sup> Our results suggest another role of hypoxia in the pathogenesis of nasal inflammation; hypoxia alters the barrier function of human nasal epithelial cells. This action may contribute to increases of bacterial or viral infections.<sup>30</sup> Also, HIF-1 $\alpha$  expression has been shown to be associated with the pathogenesis of nasal polyps.<sup>31</sup> Therefore, it is quite possible that the examination of differences in the expressions of junction complex molecules between patients with and without polyps may yield interesting results.

A previous study showed that hypoxic conditions (10% O<sub>2</sub>) do not change the proliferative capacity of nasal polyp-derived fibroblasts, whereas anoxia (0% O<sub>2</sub>) leads to a 40% reduction in cellular proliferation. This suggests that different oxygen concentrations can induce different nasal inflammatory reactions.<sup>12</sup> In the present study, we set the hypoxic condition as 1% O<sub>2</sub>, because the expression of HIF-1 $\alpha$  was not detectable at oxygen concentrations higher than 5% (data not shown). Further studies using various oxygen concentrations would be helpful to understand the pathological response that can be induced by hypoxia.

Our study has several limitations. First, we used a limited number of patient samples to assess ZO-1 and E-cadherin expression under hypoxic conditions *in vivo*. In addition to hypoxic conditions, the sinus mucosa reflects underlying inflammatory conditions. Therefore, we concluded that the expressions of ZO-1 and E-cadherin were decreased in the totally obstructed sinus mucosa compared with partially obstructed sinus mucosa. Second, we analyzed only the expression levels of ZO-1 and E-cadherin, because they are representative components of tight and adherens junctions, respectively, and their expressions in the upper airway epithelium have been previously reported.<sup>4,32,33</sup> Tight and adherens junction complexes are composed of many molecules, and there is a possibility that other junction complex molecules may be regulated differently under hypoxic conditions. Therefore, the expression of other junction complex molecules should also be evaluated after hypoxia treatment. Third, our *in vitro* experiments were performed under 1% O<sub>2</sub>, which is not a representative of the oxygen concentration in the obstructed inflammatory sinus *in vivo*. Although pO<sub>2</sub> has been shown to be lower in the opacified maxillary sinus than in the non-opacified maxillary sinus,<sup>13</sup> the *in vivo* oxygen concentration of the totally obstructed maxillary si-

nus has not been clarified. Interestingly, subepithelial blood vessels also supply oxygen to the totally obstructed maxillary sinus. Further studies on concentration of oxygen in the totally obstructed maxillary sinus would be helpful in investigating the role of hypoxia in the pathogenesis of chronic sinusitis.

In conclusion, our results suggest that exposure to hypoxic conditions down-regulates junction complex molecules and increases TER values, implying the disruption of normal barrier function of nasal epithelia. Although there are some limitations in our study, the findings described herein are the first to suggest a relationship between hypoxia and barrier dysfunction in the upper airway. Further studies on other molecules that comprise adherens and tight junction complexes would reveal the importance of hypoxia in mechanical barrier dysfunction in the upper airway epithelium.

## ACKNOWLEDGEMENTS

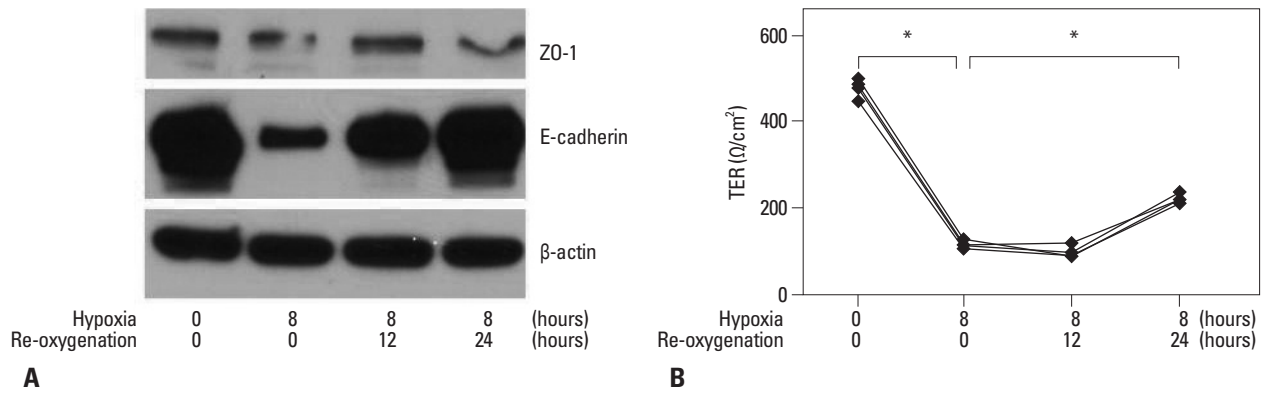
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**Supplementary Fig. 1.** Re-oxygenation after hypoxic incubation restored the barrier function in NHNE cells. (A) NHNE cells were incubated under hypoxic conditions for 8 hours, and then re-oxygenated for 0, 12, 24 hours. Western blotting was performed to assess the expressions of ZO-1, and E-cadherin. (B) NHNE cells were incubated under hypoxic conditions for 8 hours, and then re-oxygenated for 0, 12, 24 hours. TER values were measured immediately. The mean TER values of control, hypoxia, re-oxygenation for 12 hours after hypoxia, and re-oxygenation for 24 hours after hypoxia were  $479.97 \pm 21.45$ ,  $114.50 \pm 9.53$ ,  $99.50 \pm 14.38$ , and  $221.75 \pm 11.95$ , respectively ( $n=4$ ,  $*p < 0.01$ ). TER, trans-epithelial resistance; NHNE, normal human nasal epithelial.