Mechanism of hypoxia-induced hypersecretion in paranasal sinusitis

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Mechanism of hypoxia-induced hypersecretion in paranasal sinusitis

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ABSTRACT

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Excessive mucus production and hypersecretion characterize various upper airway diseases. In sinusitis, the expression of major respiratory mucin genes, such as MUC5AC, MUC5B is increased. However, the primary mechanisms leading to mucus hypersecretion in sinusitis are not well known. Hypoxia due to occlusion of sinus ostium is known to be one of the major pathologic mechanisms of the sinusitis. However, there has been no report about the mechanism of hypoxia-induced hypersecretion of mucus in sinusitis. This study is aimed to identify whether induces mucus hypersecretion in upper airway epithelia and to elucidate the mechanism of hypoxia-induced hypersecretion in upper airway epithelia.

Here we show that expression of mRNA of MUC5AC is a function of time under hypoxia in normal human nasal epithelial cells. Our results also show that the expression of MUC5AC mRNA was increased by transfection of mammalian expression vector encoding HIF-1α, a major transcription factor for hypoxia, even under normoxic condition in human lung mucoepidermoid carcinoma cell lines (NCI-H292 cells). Moreover, the induced expression of MUC5AC mRNA by

hypoxia was down-regulated by transfection of siRNA for HIF-1α. The luciferase assay for MUC5AC promoter demonstrated increased reporter activity under hypoxic condition, however the mutation of the putative HRE in MUC5AC promoter attenuated the reporter activity. We also confirmed the binding of over-expressed HIF-1α and hypoxia-response element (HRE) in the promoter of MUC5AC by electromobility shift assay. In human sinusitis mucosa, we found the overexpression of HIF-1α and MUC5AC compared to normal sinus mucosa.

Hypoxia upregulates MUC5AC expression by binding of HIF-1α to HRE in the promoter of MUC5AC. In conclusion, hypoxia upregulates mucin gene expression and secretion by signaling using HIF-1α in the airway epithelia and hypoxia might be a pathophysiologic mechanism of hypersecretion in airway disease such as sinusitis.

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I. INTRODUCTION

The epithelial surface of mammalian respiratory, gastrointestinal and reproductive tracts is coated by mucus, which is a mixture of water, glycoproteins, proteins and lipids. Mucus provides a protective barrier to epithelial compartment against pathogens and toxins. Mucus belongs to innate defensive system in mucosal immune function. Mucins are the major molecular constituents of mucus and have been implicated in health and disease. Mucin family is composed of secretory mucins and membrane-tethered mucins that integrated into the cell membrane.[1] Twenty human mucin genes are deposited in GenBank. Among them, MUC5AC and MUC5B are two major airway mucins.[2] In airway tissues from healthy individuals, goblet cells typically express and secret MUC5AC mRNA and protein, while glandular mucosal cells express and secrete MUC5B mRNA and protein.[3]

Mucus hypersecretion is known to be a major factor in the pathogenesis of the sinus disease such as paranasal sinusitis. Other reports found that levels of

MUC5AC and MUC5B mRNA in chronic rhinosinusitis were significantly increased compared with those in normal sinus mucosa. It has been reported that many infectious mediators and inflammatory/immune response mediators activate the transcription of the mucin genes.[4]

In the pathophysiologic mechanism of sinusitis, hypoxia in the sinus cavities is considered as a major pathogenic factor that causes initiation and progression of the disease.[5] This hypoxia reflects occlusion of the sinus ostium which results in failure of transepithelial oxygenation, nonvascularized exudates, and the tendency of inflammatory hyperplasia to exceed neovascularization.[6] The severity of sinus mucosal disease which correlates with opacity on computed tomography scan is reported to be related to the level of oxygen in the sinuses of patients with acute sinusitis.[7] In chronic sinusitis, the tissue hyperplasia and polyp growth can overwhelm neovascularization, further impacting local tissue hypoxia.[8] When the bulk of tissue and secretion becomes great enough, the sinuses become occluded and oxygenation of the sinus cavity is decreased.

It is believed that hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix transcription factor of the PAS family[9] plays an essential role in the cellular response to hypoxic stress by transcriptional activation of various hypoxia-inducible genes, such as those encoding erythropoietin,[10,11] vascular endothelial growth factor,[12] glycolytic enzymes,[13] glucouse transporters,[13] inducible nitric-oxide synthase,[14] heme oxygenase-1,[15] and transferrin,[16] in order to maintain oxygen homeostasis. HIF-1 is a heterodimer which is composed of an alpha and a beta subunit,[17] the latter being a constitutively-expressed aryl hydrocarbon receptor (ARNT).[18] The activity of the HIF-1 is primarily determined by hypoxia-induced stabilization of HIF-1α which becomes rapidly degraded through the ubiquitin-proteasome pathway.[19] In hypoxia, stabilized HIF-1α dimerizes with HIF-1β and binds to the hypoxia-response element (HRE) in order to recruit the transcription coactivator p300/CBP onto the promoter of hypoxia-responsive genes for

transcriptional activation. HRE is usually located at the proximal promoter (~100 bp) which contains one or more of the HIF-1 binding sites (consensus sequence 5'-[A/G]CGTG-3').[20] Mutation of HRE results in the loss of transcriptional response to hypoxia.[21] The presence of an HIF-1 binding site is necessary but not sufficient for hypoxia-response-element function.[21,22]

We noticed that the promoter region of the MUC5AC gene contains a sequence very similar to the consensus HRE within a 70 bp sequence upstream of the transcriptional start site. Therefore, in this study, we aimed to identify if hypoxia induced mucus hypersecretion and to elucidate the mechanism of hypoxia-induced hypersecretion in upper airway epithelia.

II. MATERIALS AND METHODS

1. Cell culture and hypoxia treatment

Passage-2 normal human nasal epithelial (NHNE) cells were prepared as described previously.[23] Passage-2 NHNE cells (1 x 10^5 cells) were seeded in 0.5 ml of culture medium on 24.5 mm, 0.45 μm pore size, Transwell-clear (Costar Co., Cambridge, MA, USA) culture inserts. Cells were cultured in a 1:1 mixture of bronchial epithelial growth medium (BEGM): Dulbecco's modified Eagle's medium (DMEM) containing all supplements. The cultures were grown submerged for the first 9 days, during which time the culture medium was changed on Day 1 and every other day thereafter. An air-liquid interface (ALI) was created on Day 9 by removing the apical medium and restricting the culture feeding to the basal compartment. Following ALI creation the culture medium was changed daily. The cultured epithelial cells were treated with hypoxia on Day 16 of culture.

The human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from the American Type Culture Collection (CRL-1848: Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum in the presence of penicillin-streptomycin at 37°C in a humidified chamber with 5% CO_2. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline and recultured in RPMI with 0.2% fetal bovine serum. Hypoxic condition was produced by Forma 1029 anaerobic chamber (Thermo Fisher Scientific, Waltham, MA, USA) containing a gaseous mixture of 95% N_2 and 5% CO_2.

2. RNA isolation and reverse-transcriptase PCR

Total RNA was extracted from cells using TRizol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Reverse transcription reactions were performed with 1 μg of total RNA, random hexamer

(Applied Biosystems, Foster City, CA, USA), AMV reverse transcriptase (Applied Biosystems), and RNase inhibitor (Applied Biosystems) in a final volume of 20 µl. The reverse transcription step was performed for 10min at room temperature and then 60 min at 42°C and 5 min at 95°C. RT-PCR performed with a MyCycler (Bio-Rad, CA, USA) using the primer listed in Table 1. PCR parameters used involved 24 cycles as follows: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and polymerization at 72°C for 30 sec. The PCR products were run in a 2% agarose gel and visualized with ethidium bromide under a transilluminator.

3. Real-time quantitative PCR

Real-time quantitative PCR analysis of MUC5AC gene expression was carried out with an Appylied Biosystems 7300 Fast Real-Time PCR system SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a total volume of 20 µl which included 10 µl of 2X SYBR Green PCR Master Mix, 300 nM of each primer, and 1 µl of previously reverse-transcribed cDNA template. Primers are listed in Table 1. The thermocycler parameters were 50°C for 2 min, and 95°C for 20 sec, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. All reactions were performed in triplicate. The relative quantity of MUC5AC mRNA was obtained using the comparative cycle threshold method and was normalized using β2-microglobulin as an endogenous control.

Table 1. Primer used for PCR

Gene name	Primer sequences		Product size
MUC5AC (RT-PCR)	Forward	GTG GAA CCA CGA TGA CAG C	818 bp
	Reverse	TCA GCA CAT AGC TGC AGT CCG	
MUC5AC (Real-time PCR)	Forward	CAG CCA CGT CCC CTT CAA TA	66 bp
	Reverse	GAA CCG CAT TTG GGC ATC C	
β2-Micrglobulin (RT-PCR)	Forward	TCG CGC TAC TCT CTC TTT CTG G	334 bp
	Reverse	GCT TAC ATC TCT CGA TCC CAC TTA A	
β2-Micrglobulin (Real-time PCR)	Forward	CGC TCC GTG GCC TTA GC	67 bp
	Reverse	GAG TAC GCT GGA TAG CCT CCA	
HIF-1α (RT-PCR)	Forward	GCA GCC AGA TCT CGG CGA AG	319 bp
	Reverse	CTG TGT CCA GTT AGT TCA AAC TG	

4. Western blot analysis

After hypoxia treatment, the NHNE cells or NCI-H292 cells were lysed with cell extraction buffer (1 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1%Triton X-100, 10% glycerol 0.1 % SDS) (Invitrogen, Carlsbad, CA, USA) included 1 mM PMSF and 10 % sodium deoxycholate. Equal amounts of whole cell lysates were resolved by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5 % skim milk in Tris-buffered saline (50 mM Tris-Cl pH 7.5, 150 mM NaCl) for 2 hr at room temperature. This blot was then incubated overnight with primary antibody (HIF-1α) (1:1000) (BD Bioscience, San Jose, CA, USA) in TTBS (0.5 % Tween 20 in Tris-buffered saline). After washing with TTBS, the blot was further incubated for

1 hr at room temperature with anti-mouse secondary antibody (Cell Signaling, Danvers, MA, USA) in TTBS and then visualized by using the ECL system (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

5. HIF-1α siRNA preparation and transfection

Specific siRNA against HIF-1α was used to suppress their respective expressions. HIF-1α siRNA oligonucleotides (Stealth™ siRNA) were synthesized by Invitrogen (Invitrogen, Carlsbad, CA, USA). We screened HIF-1α mRNA (GenBank NM_001530) and selected potential siRNA sequences with high values of knock-down probability. The siRNA sequences were 5'-GUG GUU GGA UCU AAC ACU A-3'. Stealth RNAi negative control duplex (Medium GC, Invitrogen) was used as a siRNA negative control. siRNA transfection into NCI-H292 cells was carried out with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Ten pmole of each siRNA and 2 μl lipofectamine ™ were mixed with RPMI without serum and antibiotics and then transfection was performed onto 6-well NCI-H292 cells when the cells reached 30 - 50% confluence. This procedure did not affect cell viability. The same procedure was performed with control siRNA.

6. Transient transfeciton of mammalian expression vector for HIF-1α

NCI-H292 cells were seeded into six-well plates and cultured for 24 hr before transfection. Cells were transfected with 1 μg of PCMV-HIF-1α containing the full length HIF-1α cDNA (HIF-1α overexpression plasmid), or PCMV-NN (negative control; empty vector) for 24 hr using FuGENE6 Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Cells were then treated with hypoxia and assayed by RT-PCR, real-time PCR, or Western blot analysis. Dr. Koh EM at Sungkyunkwan University (Seoul, Korea) provided PCMV-HIF-1α overexpression plasmids.[24]

7. Transient transfection and luciferase analysis

NCI-H292 cells were transiently transfected with the plasmids for MUC5AC promoter luciferase assay with pGL3-basic, pGL3-MUC5AC promoter (-1376/+4), pGL3-mutated MUC5AC promoter (-1376/+4) using FuGENE 6 transfection reagent (Roche Applied Science, Bagel, Switzerland) according to the manufacturer's instructions in normoxia or hypoxia condition. After transfection of reporter, the cells were incubated under the defined conditions for 6 hr and assayed for luciferase activity, using a luciferase assay system (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. Beta-galactosidase activity was also assayed to standardize the transfection efficiency of each sample. Mutation of the putative regulatory elements that start from -65 bp upstream of MUC5AC gene was generated by mutagenic PCR (5'-A**CG**TG-3' → 5'-A**AA**TG-3', -65/-61).

8. Electrophoretic mobility shift assays (EMSAs)

The binding activity of HIF-1α and HRE on MUC5AC promoter was detected by EMSA. The single-stranded oligonucleotide sequence of 5'-ccc acc **cac gtg** aag cac g-3', 3'-cgt gct tc**a cgt g**gg tgg g-5', which corresponds to the HIF-1α binding site, was end labeled with biotin (100 pmole, Bioneer, Daejeon, Korea). Cells were then resuspended in cell homogenization buffer containing 0.05% (v/v) Nodient P-40 and then homogenized. Next, nuclei were pelleted and resuspended in cell resuspension buffer (40 mM HEPES pH 7.9, 0.4 M KCl, 1 mM dithiothreitol, 10% (v/v) glycerol, 0.1 mM phenlmethylsulfonylfluoride, 0.1 % (w/v) aprotinin and 0.3 M NaCl). This nuclerar extract was then centrifuged at 14000 rpm for 10 min at 4°C, and the supernatant was aliquoted and stored at -70°C. Five μg of nuclear extract were incubated at room temperature for 20 min with biotin-labeled–HRE oligonucleotide in Chemiluminescent Nucleic Acid Detection Kit (Thermo,

Rockford, IL, USA). Oligo-nuclear extract complex were separated by electrophoresis through 6% non-denaturing polyacrlyamide gels in 0.5X Tris borate-EDTA (TBE) buffer. The gel was transferred to Biodyne B pre-cut modified Nylon membranes (Thermo, Rockford, IL, USA). Blotting were used Chemiluminescent Nucleic Acid Detection Kit (Thermo, Rockford, IL USA).

9. Immunohistochemical staining for MUC5AC and HIF-1α

Human mucosal samples were obtained from the maxillary sinus antral mucosa of the patients with sinusitis confirmed by CT and normal human sample were obtained from normal-appearing ethmoid mucosa of that subjects. The normal sample and sinusitis samples were fixed with 10% formaldehyde solution for 24 hr and then dehydrated and embedded in paraffin. Paraffin blocks were sectioned into 4 μm-thick slices and fixed. After deparaffinization and rehydration, slides were incubated in antigen retrieval solution (Tris-EDTA, pH 9.0) for 20 min at 90-95°C. To block endogenous peroxidase, slides were treated with 0.3 % H_2O_2 for 15 min at room temperature. Slides were blocked with 10% normal serum with 1% BSA in TBS for 2 hr at room temperature, and then incubated overnight at 4°C with a monoclonal mouse antibody against human MUC5AC (1:100 Jackson Immune Research, Sacramento, CA, USA) and HIF-1α (BD Bioscience, San Jose, CA, USA). The slides were then incubated with HRP-conjugated goat anti-mouse IgG (1:200; Jackson Immune Research) in antibody diluent solution (DAKO, Glostrup, Denmark) at room temperature and counterstained with hematoxylin (Merck, Darmstadt, Germany).

10. Statistical analysis

Data are presented as means $\pm$ SD of at least three independent experiments. Where appropriate, statistical differences were assessed by Wilcoxon Mann–Whitney tests. A p-value less than 0.05 was considered statistically significant.

III. RESULTS

1. Messenger RNA expression of MUC5AC according to hypoxic stimulation in NHNE cells

To determine whether hypoxic stimulation induces the expression of MUC5AC gene in NHNE cells, mRNA expression of MUC5AC in NHNE cell was analysed by real-time PCR after incubating the NHNE cells in the hypoxic chamber with 95% N_2, 5% CO_2 in time-dependant manner. The mRNA levels of MUC5AC were measured with increasing time at 0, 1, 2, 4, 6 and 12 hr after hypoxic treatment. Compared with non-treated cells, the mRNA levels of MUC5AC were increased as a function of time in the cells under hypoxic treatment. These results indicate that MUC5AC expression in NHNE cells is induced by hypoxia (Fig. 1).
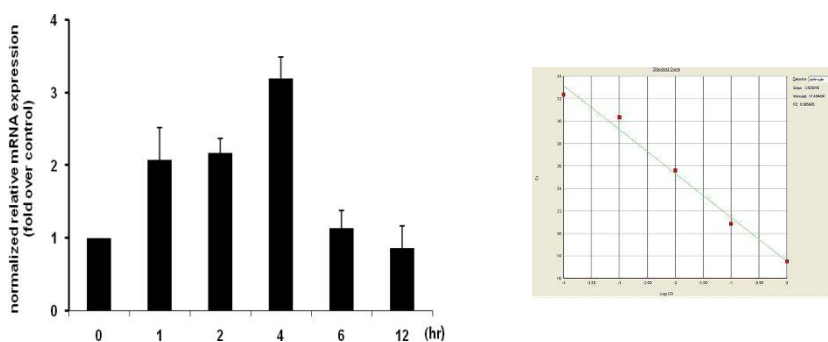

Figure 1. mRNA expression of MUC5AC according to hypoxic stimulation in NHNE cells. NHNE cells were exposed to 95% N_2, 5% CO_2 in the hypoxic chamber for 0 - 12 hr. Total RNA of the NHNE cells was isolated and the expression level of mRNA of MUC5AC was analyzed by real-time PCR. The expression of MUC5AC mRNA was increased under the hypoxic condition as a function of time under hypoxic treatment having its peak in 4 hr.

2. Loss-of-function study with siRNA for HIF-1α in NCI-H292 cells

To determine whether HIF-1α is involved in the regulation of MUC5AC by hypoxia, siRNA for HIF-1α or control siRNA was transiently transfected into the human mucoepidermoid carcinomal cell lines (NCI-H292 cells). And then, these cells were treated with hypoxia for 12 hr. Initially, western blot analysis was carried out to confirm the expression of HIF-1α. We could confirm the effect of siRNA for HIF-1α with the reduction of over-expressed HIF-1α protein by siRNA for HIF-1α compared to those of control siRNA (Fig. 2A). By the transfection of siRNA for HIF-1α, hypoxia-induced over-expression of MUC5AC mRNA was decreased with RT-PCR (Fig. 2B) and also with real-time PCR (Fig. 2C).
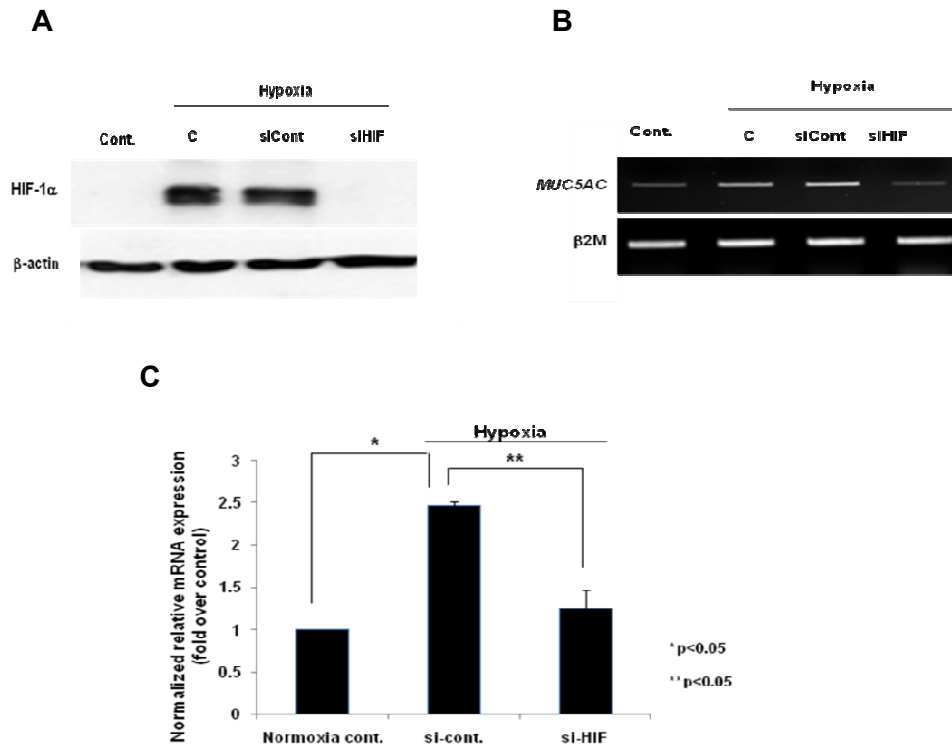
A

B

C

Figure 2. Loss-of-function study with siRNA for HIF-1α in NCI-H292 cells. NCI-H292 cells transient-trnasfected with siRNA for HIF-1α or control siRNA were incubated in hypoxic chamber with 95% N$_2$, 5% CO$_2$ for 12 hr. **A:** Induced expression of HIF-1α protein by hypoxia was decreased by transfection of siRNA for HIF-1α compared to those of control siRNA. **B:** By transfection of siRNA for HIF-1α, induced expression of MUC5AC mRNA was remarkably decreased accordingly using RT-PCR analysis. C: This finding was also confirmed with real-time PCR analysis.

3. Gain-of-function study with mammalian expression vector for HIF-1α in NCI-H292 cells

To examine the functional role of HIF-1α in MUC5AC gene expression, we used mammalian expression vector for HIF-1α in NCI-H292 cells. We transiently transfected the cells with vector containing HIF-1α (pCMV-HIF-1α: generous gift from Dr. Koh EM, Sungkyunkwan University, Seoul, Korea). Initially, western blot analysis was carried out to confirm the expression of HIF-1α. The expression was increased in the cells tranfected with HIF-1α expression plasmids compared to the cells with empty vectors even in normoxic condition (Fig. 3A). Messenger RNA of MUC5AC was measured by RT-PCR which showed that transfection of plasmids encoding HIF-1α increased the expression of MUC5AC mRNA in the NCI-H292 cells (Fig. 3B). This was also confirmed in real-time PCR experiments (Fig. 3C)
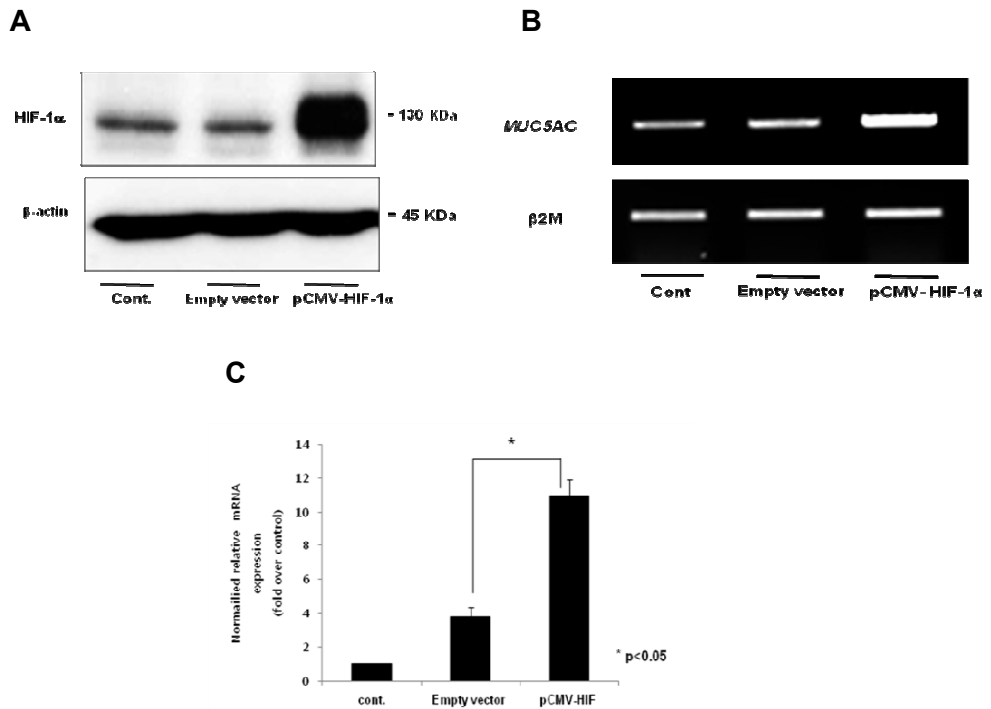
Figure 3. Gain-of-function study with mammalian expression vector for HIF-1α in NCI-H292 cells. **A:** pCMV vector encoding HIF-1α or pCMV vector without HIF-1α were transiently transfected into NCI-292 cells. The expression of HIF-1α was increased in the cells transfected with pCMV-HIF-1α vector compared to the cells with empty vectors even in the normoxic condition. **B:** mRNA expression of MUC5AC. The increase of MUC5AC mRNA was noted in the cells with pCMV vectors encoding HIF-1α compared to the cells with empty vectors. **C:** This was also confirmed in real-time PCR experiments.

4. Luciferase assay of MUC5AC promotor in hypoxia

To figure out whether putative HRE of MUC5AC promoter plays a important role in hypoxia-induced MUC5AC expression, luciferase assay with mutagenesis of putative HRE was carried out. The putative HRE is located 61~65bp upstream of the MUC5AC gene, of which sequence is 5'-ACGTG-3'. By mutagenic PCR, putative HRE sequence was mutated to 5'-AAATG-3'. These wild-type or HRE-mutated MUC5AC promoters were inserted into pGL3 plasmids, which have the luciferase reporter gene. NCI-H292 cells were transfected with pGL3 vectors containing wild-type MUC5AC promoter, pGL3 vectors with HRE-mutated MUC5AC promoter, and pGL3-basic vectors, respectively. After treatment of hypoxia with 95% N_2, 5% CO_2 for 6 h, the relative luciferase activity in the cells with pGL3-wild-type MUC5AC promoter was increased by 5.11-fold compared to the HRE-mutated group and by 6.44-fold compared to the pGL3-basic vector group. In normoxia, the luciferase activity in the cells with plasmids encoding wild-type MUC5AC promoter was increased compared to the groups with either HRE-mutated promoter or pGL3-basic vector. Compared with the normoxic condition, the luciferase activity was increased by 1.8-fold in the cells with wild-type MUC5AC promoter in the hypoxic condition. Hypoxic stimulation did not increase the activity of luciferase reporter when the MUC5AC promoter HRE was mutated (Fig. 4). This result implicates that putative HRE located on MUC5AC promoter plays a important role in hypoxia-induced MUC5AC expression.
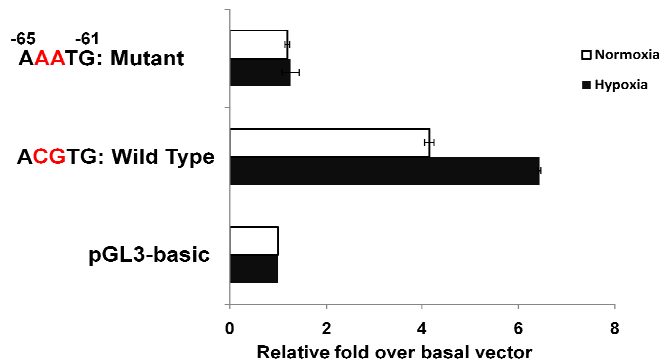
Figure 4. Luciferase assay of MUC5AC promoter. NCI-H292 cells were transfected with pGL3-basic vectors, pGL3 vectors encoding wild-type MUC5AC promoter, or pGL3 vectors encoding HRE-mutated MUC5AC promoter. After cells were incubated in the hypoxic chamber with 95% N_2, 5% CO_2 for 6 hr, the luciferase activity was increased by 1.8-fold in the cells with wild-type MUC5AC promoter in the hypoxic condition compared with the normoxic condition. Hypoxic stimulation did not increase the activity of luciferase reporter when the MUC5AC promoter HRE was mutated.

5. HIF-1α binding to the MUC5AC promoter in response to hypoxia

To analyze the DNA binding activity of hypoxia-induced-HIF-1α, we performed an EMSA assay using the nuclear extracts of hypoxia-treated NHNE cells (Fig. 5). The activities of HIF-1α specific HRE-oligonucleotides increased remarkably in response to hypoxia. These results indicate that induced HIF-1α binds to a HRE in the MUC5AC promoter.
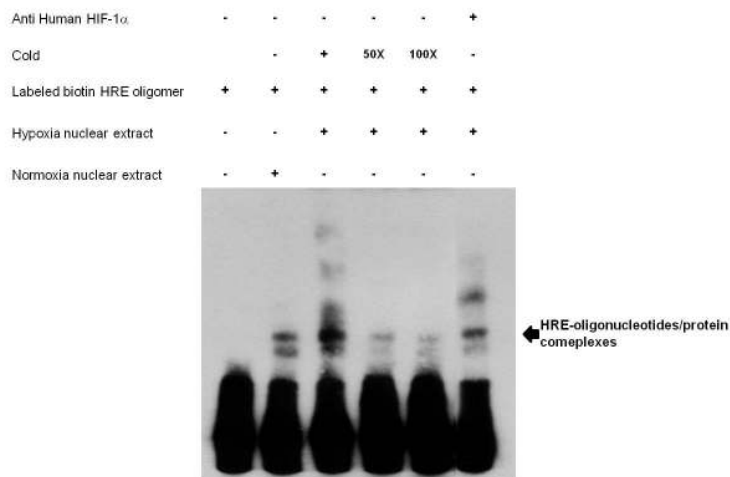

Figure 5. HIF-1α binding to the HRE in response to hypoxia. Nuclear protein extract from hypoxia-treated NHNE cells was subjected to an electromobility shift assay. The activities of HIF-1α specific HRE-oligonucleotides increased remarkably in response to hypoxia.

6. HIF-1α protein expression in normal vs. sinusitis mucosa

Based on our result, we wanted to examine the HIF-1α expression in human samples. We conducted Western blot analysis with mucosal sample of sinusitis patients vs. normal ethmoid mucosal samples of same subject. The results showed that HIF-1α protein was obviously over-expressed in the sinusitis samples compared to normal samples (Fig. 6A). In densitometric comparison, expression of HIF-1α was 4.4-fold higher in sinusitis samples compared to normal samples (Fig. 6B). We also confirmed induced expression of HIF-1α protein in sinusitis samples with immunohistochemical staining (Fig. 6C).

A

Patient #1   Patient #2   Patient #3   Patient #4
N   S   N   S   N   S   N   S

HIF-1α

α-tubulin

B

C

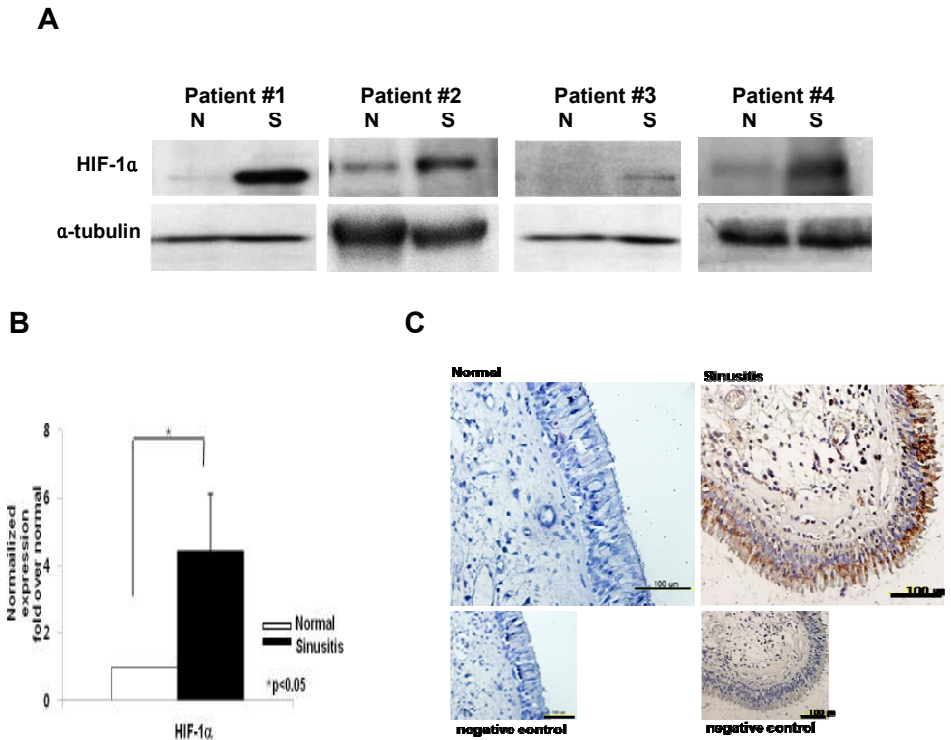**Figure 6.** HIF-1α protein expression in normal vs. sinusitis mucosa. **A:** HIF-1α protein was obviously over-expressed in the sinusitis samples compared to normal samples. **B:** In densitometric comparison, expression of HIF-1α was 4.4-fold higher in sinusitis samples compared to normal samples. **C:** This result was also confirmed with immunohistochemical staining for HIF-1α in sinusitis samples.

7. MUC5AC expression in normal vs. sinusitis mucosa

We also investigated the expression of MUC5AC, target protein for HIF-1α, in normal versus sinusitis mucosa by immnohistochemical staining (Fig. 7). MUC5AC was highly expressed throughout the epithelium of sinusitis, where the hypoxia is supposed to be present by occlusion of sinusus ositum.
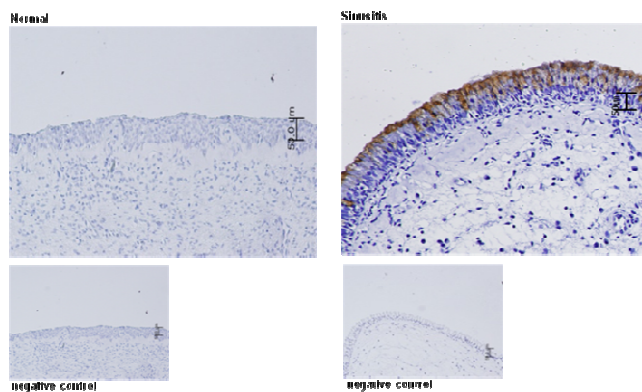

Figure 7. MUC5AC expression in normal vs. sinusitis mucosa. We observed the upregulated MUC5AC expression in sinusitis mucosa, where the hypoxia is supposed to be present by occlusion of sinusus ositum.

IV. DISCUSSION

This study was performed based on the presumption that the hypoxic condition within the sinuses can directly induce the hypersecretin of mucin which is one of the major pathophysiologic mechanisms of sinusitis. Although hypoxia is known as a potent stimulant for inflammation[8] and tissue remodeling[25] that can contribute to sinus diseases, no studies have been performed to investigate the effect of hypoxia on mucin secretion in airway epithelial system. Only MUC3 gene in intestinal epithelium is known to be regulated by hypoxic stimulation.[26]

This study demonstrated that the MUC5AC gene is induced by hypoxia in normal human nasal epithelial cells (Fig. 1). The transcriptional initiation of MUC5AC occurred after the cells were exposed to hypoxic conditions. One possible mechanism by which hypoxia can regulate MUC5AC gene expression is through the transcription factor HIF-1. HIF-1 is heterodimeric proteins composed of an oxygen regulated α subunit and an oxygen independent β subunit.[27] The α subunits are continuously transcribed and translated. Under normoxic conditions, two prolines in the α subunit are hydroxylated, which enables the protein to be ubiquinated by the von Hippel-Lindau tumor suppressor and degraded in the 26S proteosome.[28] When oxygen levels decrease, the α and β subunits heterodimerize and bind to specific elements (hypoxia-response element) in promoters of various genes to induce transcription. In this study, although the mRNA expression of HIF-1α was not influenced by hypoxic stimulations (not shown), the protein level of HIF-1α was increased with time under hypoxic conditions (not shown), which suggests post-translational regulation of HIF-1α protein.

We found that the MUC5AC promoter region contains a putative HRE. The HRE of the MUC5AC promoter exhibits core sequence ACGTG that are similar to the sequences found in the promoters of transferrin or lactate dehydrogenase. To evaluate the importance of HIF-1α for induction of MUC5AC in

hypoxic condition, we conducted loss-of-function test using siRNA for HIF-1α in hypoxic condition. By knock-down of HIF-1α gene expression, MUC5AC mRNA expression was reduced accordingly (Fig. 2). This result indicates that transcription factor HIF-1a is important factor for induction of MUC5AC by hypoxia.

To evaluate the role of HIF-1α, we also conducted the gain-of-function test. HIF-1α expression vectors were transfected in human lung mucoepidermoid carcinoma cell lines. The MUC5AC promoter activity was enhanced by increased expression of HIF-1α even in the normoxic condition. However, compared to the increased amount of HIF-1α protein (Fig. 3A), the increased MUC5AC mRNA levels in the cells with overexpression of HIF-1α did not appear to be sufficient (Fig. 3B, 3C). It may be suggested that additional overexpression of HIF-1β could contribute to higher activity of MUC5AC promoter as observed in other studies in which co-transfection of HIF-1α and HIF-1β resulted in higher activity of transferrin receptor promoter than HIF-1α alone.[29] These results suggest that the MUC5AC gene contains an enhancer regulated by HIF-1. Moreover, our studies demonstrated that the wild-type but not HRE-mutated MUC5AC promoter conferred inducibility to the luciferase reporter gene under the hypoxic condition in NCI-H292 cells (Fig. 4). However, the difference of relative activity between the cells incubated in the hypoxic and normoxic conditions was not very significant, meaning that there may be another mechanism involved in activating the MUC5AC gene mediated by the putative HRE.

Because putative HRE-mutated MUC5AC promoter attenuated the reporter activity, putative HRE in the MUC5AC promoter can be a strong candidate for a functional HRE. To confirm whether HIF-1α can bind to the putative HRE in the MUC5AC promoter, EMSA was performed. Our study reveals that hypoxia can contribute to the sinus disease by directly regulating MUC5AC secretion via the activation of HIF-1α.

MUC5AC is the one of the major secreted mucin in sinusitis. Considering that a marked increase of MUC5AC and MUC5B secretion was reported in sinus diseases and the mean level of MUC5AC expression was higher compared to MUC5B in sinus mucus secretion,[30] the role of hypoxic stress in the pathophysiology of sinusitis could be more accentuated. Therefore, in the management of upper airway diseases like sinusitis, the mechanical or pharmacological restoration of ventilation would be crucial.

Ⅴ. CONCLUSION

Our study revealed that the expression and secretion of MUC5AC, which is the major component of mucus in sinusitis, can be increased with hypoxic stimulation in NHNE cells. HIF-1α is the well-known transcription factor which activates several genes responding to hypoxic stimulation. The alteration of the activity of HIF-1α by overexpression or siRNA of HIF-1α affected the expression of MUC5AC. Mutation of the putative HRE in the MUC5AC promoter attenuated the reporter activity in promoter luciferase assay. Hypoxia-induced MUC5AC over-expression was mediated by binding of HIF-1α to HRE in the promoter of MUC5AC.

In conclusion, hypoxia upregulates mucin gene expression and secretion by signaling using HIF-1α in the airway epithelia and hypoxia might be a pathophysiologic mechanism of hypersecretion in airway diseases such as sinusitis.

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ABSTRACT (IN KOREAN)

저산소에 의한 부비동염의 과분비 기전규명

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과도한 점액 생성 및 과분비는 여러 상기도 질환의 특징이다. 부비동염에서 중요 점액인 MUC5AC와 MUC5B의 발현이 증가되어 있다고 알려져 있다. 하지만 부비동염에서 어떠한 기전에 의해 점액의 과분비가 유도되는 지는 아직 잘 알려지지 않았다. 부비동 개구부의 폐쇄에 의한 저산소증은 부비동염의 가장 중요한 병인으로 알려져 있다. 하지만 부비동염에서 저산소증에 의해 유도되는 과분비 기전은 아직 잘 알려지지 않았다.

이 연구의 목적은 저산소에 의해 점액의 과분비가 일어나는지, 그렇다면 저산소에 의해 유도되는 점액 과분비의 기전은 어떠한지를 알아보고자 하였다.

본 연구자는 배양된 정상사람 코점막상피세포에서 시간에 따른 저산소 처치에 의해 MUC5AC의 유전자 발현이 증가함을 보였다. 또한 이러한 증가가 저산소의 중요 전사인자인 HIF-1α의 siRNA 처치에 의해 감소함을 보였고, 과발현 벡터를 transfection함으로써 정상 산소조건에서도 MUC5AC 유전자의 과발현을 유도할 수 있었다.

Luciferase 실험을 통해 저산소 환경에서 MUC5AC의 reporter 활성이 증가함을 관찰할 수 있었는데, 이는 MUC5AC의 promoter에 존재하는 HRE의 mutation을 통해 감소됨을 확인할 수 있었다. 연구자는 과발현된 HIF-1α 전사인자와 MUC5AC promoter에 존재하는 HRE와의 결합을 EMSA 방법을 통하여 확인할 수 있었다. 사람 부비동염 샘플에서 HIF-1α 단백과 MUC5AC 단백의 과발현을 면역화학염색을 통해 확인할 수 있었다.

이 연구를 통해 저산소가 HIF-1α와 MUC5AC의 promoter에 존재하는 HRE와의 결합을 통해 MUC5AC의 발현을 증가시킴을 알 수 있었다. 결론적으로 저산소는 HIF-1α 전사조절에 의해 점액의 발현 및 분비를 조절함을 알 수 있었고, 저산소가 부비동염과 같은 기도질환에서 과분비를 일으키는 중요 병인으로써의 역할을 함을 알 수 있었다.