The Signal Pathway of Platelet Derived Growth Factor-induced *MUC8* overexpression in Human Airway Epithelium

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The Signal Pathway of Platelet Derived Growth Factor-induced *MUC8* overexpression in Human Airway Epithelium

Directed by Professor Joo-Heon Yoon

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The certifies that the Doctoral Dissertation of Hyun Jik Kim is approved

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감사의 글

본 논문을 완성하기까지 여러모로 부족한 저를 언제나 큰 사랑으로 헌신적 지도를 해주신 윤 주헌 교수님께 진심으로 감사드립니다. 선생님의 지도와 관심 그리고 질책이 없었더라면 제가 대학원 박사 과정을 무사히 마치지 못했을 것으로 생각됩니다. 또한 바쁘신 와중에서도 지도 편달과 성원을 베풀어주신 김 창훈 선생님과 내과학 교실 김 세규 선생님, 생화학 교실 김 경섭 선생님, 이화 여대 이 원재 선생님 배 윤수 선생님께도 진심으로 감사를 드리며 본 연구가 가능하도록 도와주신 문 욱렬 선생님과 이비인후과 연구원 여러분께도 감사의 말씀을 전합니다.

모교를 떠나 외부 병원에서 근무를 하며 학위 실험을 한다는 것이 쉽지는 않았고 결과가 잘 나오지 않을 때는 많은 좌절감도 있었습니다. 외부에서 온 제가 실험과 연구를 할 수 있게 도와주신 이 정권 연세대학교 이비인후과 교실 주임교수님, 양 훈식 중앙대학교 이비인후과 교실 주임 교수님, 그리고 제가 자리를 비울 때 저의 환자들을 잘 돌보고 치료해 주신 김 경수, 김 준희, 김 희종, 박 성윤, 위 민우 선생님 등 중앙대학교 이비인후과 교실원들

께 감사드리며 마지막으로 실험과 진료를 우선으로 하고 항상 가족은 뒷전 이었는데 특히 사랑하는 김 정재 군에게 감사와 용서의 마음을 전합니다.

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It is well known that reactive oxygen species (ROS) may participate in the pathogenesis of various inflammatory airway diseases and specific enzymes, including NADPH oxidase (Nox), have unique function of generating ROS. Growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor, can enhance the intracellular ROS generation, resulting in mucus hypersecretion that is a prominent manifestation in chronic inflammatory airway diseases. The purpose of this study was to identify which mucin genes are induced by stimulation with PDGF and the enzymatic mechanism by which these genes are up-regulated in normal human nasal epithelial (NHNE) cells. The amount of PDGF was increased in the tissue of sinusitis and PDGF induced *MUC8* gene overexpression through intracellular ROS generation in NHNE cells. In addition, Nox4, a subtype of

non-phagocytic NADPH oxidase, was found to play a essential role in intracellular ROS generation and PDGF‐induced *MUC8* overexpression in NHNE cells.

Keywords : PDGF, MUC8, Nox4. Reactive oxygen species

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Ⅰ. Introduction

Oxidative injury triggered by either inhaled or locally generated reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2-), and hydroxyl radical, has been considered as harmful molecules that may be related with cancer, cardiovascular diseases, degenerative diseases and inflammatory diseases[1,2]. Several airway diseases are characterized by excessive ROS production and response to ROS that can profoundly impair the structural integrity and biological properties of bronchial epithelium[3]. There are several potential sources of ROS in phagocytic or non-phagocytic cells, including nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, xanthine oxidase, uncoupled nitric oxide (NO) synthase and the mitochondrial respiratory chain reaction[4]. However, evidences indicate that the primary form of the ROS is superoxide anion (O_2^-) and it is produced via

the one-electron reduction of O_2 by the multicomponent NADPH oxidase (Nox) system in phagocytic cells[5]. In contrast, the mechanism behind ROS generation in non-phagocytic cells remains unclear but evidence suggests that seven homologs of gp^{91phox} (Nox2), the core component of Nox, have been identified in various non-phagocytic cells (Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2)[6].

Mucus hypersecretion is the major pathological manifestation which is commonly observed in chronic respiratory diseases such as rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis and cystic fibrosis[7,8]. Mucin is a major component of this mucus in respiratory tract. MUC5AC and MUC5B has been generally recognized to be the major airway mucin genes, highly expressed in the goblet cells of human airway epithelium and the submucosal glands[9]. However, instead of MUC5AC and MUC5B, MUC8 is overexpressed in nasal polyp epithelium and is also increased by treatment with inflammatory mediators in nasal epithelial cells[10]. Therefore, MUC8 may be one of important mucin gene expressed in the chronic inflammation of human airway epithelium[10]. ROS are produced in airway epithelial cells and the identification of Nox homologues in airway epithelial cells implies the generation of ROS[11,12]. Recently, Duox1 was identified in normal human bronchial epithelial cells and shown to generate ROS[11,13,14]. Nox4 was expressed predominantly in goblet cells of normal human nasal epithelial (NHNE) cells, resulting in generation of intracellular ROS[15]. These Nox-induced ROS are thought to modulate the expression of a variety of mucin genes that are involved in mucus hypersecretion and also play a role in the airway inflammatory diseases. The regulation of genes or signal

proteins by ROS involves receptor tyrosine kinase such as platelet derived growth factor (PDGF) receptor and epidermal growth factor receptor[11,16,17].
PDGF is a potent mitogen and chemoattractant for cells of mesenchymal origin and plays an important role in cell proliferation, differentiation and chemotaxis and is involved in proliferative disorders[18,19].It consists of two chains, A and B, held together in homodimeric or heterodimeric configuration by disulfide bonds. The three isoforms of PDGF (AA, BB and AB) modulate biological responsiveness by interacting specifically with two (α and ß) cell surface tyrosine kinase receptors. α binds the A-chain and the B-chain, whereas ß binds only to the B-chain[18,20]. The PDGF ligand receptor system has strongly been implicated to play roles in a large number of normal and pathologic settings, such as embryonic development, wound healing, tumorigenesis, and atherosclerosis[21].

In the present study, we first, examined whether PDGF A or B was increased in the tissue of sinusitis patients and the correlation between overexpression of PDGF and generation of ROS in NHNE cells. We measured intracellular ROS generation after the stimulation with PDGF and examined which Nox subtype was involved in the endogenous generation of ROS in NHNE cells. Second, we investigated which mucin genes were induced by the stimulation with PDGF in NHNE cells.

II. Material and Methods

Materials

PDGF-BB, Anti-α-tubulin antibody and N^G-Monoethyl-L-arginine (NMEA)

were purchased from Calbiochem (San Diego, CA, USA). Anti-Nox4, Anti-PDGF-A, Anti-PDGF-B antibodies were purchased from Santa Cruz Biothechnology (Santa Cruz, CA, USA). N-acetyl-L-cysteine (NAC), allopurinol, dicumarol and diphenyleneiodium chloride (DPI) were purchased from Sigma Aldrich (St. Louis, MO). Control and recombinant adenoviruses encoding Nox4 construct and siRNA for Nox4 were provided by Dr. Yun Soo Bae (Ewha Womans Univ, Seoul, Korea). A sequence of 21-nuleotide residues in length (GTCAACATCCAGCTGTACCdTdT) specific to the human Nox4 cDNA(nucleotide residues, 1474-1492) was selected for synthesis of a siRNA. The depletion of endogenous Nox4 by the siRNA was confirmed by RT-PCR.

Cell culture

After approval of the study protocol by the Institutional Review Board of the Yonsei University College of Medicine, human nasal middle turbinates specimens were obtained from two healthy volunteers. The culture system used for the normal human nasal epithelial (NHNE) cells was described previously[22,23]. In brief, passage-2 NHNE cells (1 X 10^5cells/culture) were seeded in 0.5 ml of culture medium onto a 24.5-mm, 0.45-um-pore transwell-clear (Costar Co, Cambridge, MA, USA) culture insert. Cells were cultured in a 1:1 mixture of basal epithelial growth medium and Dulbecco's Modified Eagle's Medium containing all the supplements described previously[23]. Cultures were grown while submerged for the first nine days, during which time the culture medium was changed on day 1 and every other day thereafter. The air-liquid interface (ALI) was created on day 9 by

removing the apical medium and feeding the cultures from only the basal compartment. The culture medium was changed daily after the ALI was initiated, and all experiments utilized NHNE cells on 14 days after the creation of the ALI.

The human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from the American Type Culture Collection (CRL-1848; Manassas, VA). These cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 39°C in a humidified chamber with 5% CO_2. Confluent cells were washed twice with phosphate-buffered saline and recultured in RPMI 1640 with 0.2% fetal bovine serum to deprive them of serum[24].

Methods

RT-PCR

Total RNA was isolated from NHNE cells treated with PDGF-BB (5, 10, 25, 50 and 100 ng/ml) using TRIzol (Invitrogen). cDNA was synthesized with random hexamer primers (PerkinElmer Life Sciences and Roche Applied Science) using Molony murine leukemia virus-reverse transcriptase (PerkinElmer Life Sciences). Oligonucleotide PCR primers were designed based on the MUC5AC, MUC1, MUC4 and MUC8 Genbank TM sequences and Nox subunit sequences (Tables 1, 2).

Product	Cycle	Annealing Temp (°C)	Primer	Oligonucleotide Sequence
MUC5AC	32	60	Forward	CGA CAA CTA CTT CTG CGG TGC
			Reverse	GCA CTC ATC CTT CCT GTC GTT
MUC5B	35	55	Forward	CTG CGA GAC CGA GGT CAA CAT C
			Reverse	TGG GCA GCA GGA GCA CGG AG
MUC8	35	55	Forward	ACA GGG TTT CTC CTC ATT G
			Reverse	CG TTA TTC CAG CAC TGT TC
MUC4	35	55	Forward	TGG AAC CAT TTC TGC AAT CA
			Reverse	GAG GAA GGC CAT GTT GTT GT
MUC1	30	55	Forward	CTC ACC TCC TCC AAT CAC
			Reverse	GAA TGG CAC ATC ACT CAC
β2-M	23	55	Forward	CTC GCC CTA CTC TCT CTT TCT GG
			Reverse	GCT TAC ATG TCT CGA TCC CAC TTA A

Table 1. Polymerase chain reaction experimental conditions and oligonucleotide sequences.

Product	Size (bp)	Cycle	Annealing Temp (°C)	Primer	Oligonucleotide Sequence
Nox1	400	35	60	Forward	GTA CAA ATT CCA GTG TGC AGA CCA C
				Reverse	CAG ACT GGA ATA TCG GTG ACA GCA
Nox2	550	35	55	Forward	GGA GTT TCA AGA TGC GTG GAA ACT A
				Reverse	GCC AGA CTC AGA GTT GGA GAT GCT
Nox3	457	35	55	Forward	ATG AAC ACC TCT GGG GTC AGC TGA
				Reverse	GGA TCG GAG TCA CTC CCT TCG CTG
Nox4	285	32	55	Forward	CTC AGC GGA ATC AAT CAG CTG TG
				Reverse	AGA GGA ACA CGA CAA TCA GCC TTA G
Nox5	238	35	55	Forward	ATC AAG CGG CCC CCT TTT TTT CAC
				Reverse	CTC ATT GTC ACA CTC CTC GAC AGC
Duox1	106	32	55	Forward	CGA CAT TGA GAC TGA GTT GA
				Reverse	CTG GAA TGA CGT TAC CTT CT
Duox2	181	32	55	Forward	CTC TCT GGA GTG GTG GCC TAT T
				Reverse	GGA CCT GCA GAC ACC TGT CT

Table 2. Polymerase chain reaction experimental conditions and oligonucleotide sequences.

We used comparative kinetic analysis to compare mRNA levels for each gene for each set of culture conditions. The PCR products were resolved on a 2% agarose gel (FMC, Rockland, ME) and visualized with ethidium bromide under a transilluminator. When the reverse transcriptase was omitted, no PCR products were observed. This result confirms that the amplified products were from mRNA and not genomic DNA contamination. Specific amplification of

target genes was confirmed by sequencing the PCR products (dsDNA Cycle Sequencing System;GibcoBRL, Rockville, MD).

Real time-PCR

Primers and probes were designed using PerkinElmer Life Sciences Primer Express® software purchased from PE Biosystems. Commercial reagents (TaqMan PCR Universal PCR master mix, PE Biosystems) and conditions were applied according to the manufacturer's protocol. One micrigram of cDNA (reverse transcription mixture), oligonucleotides at a final concentration of 800 nM of primers, and 200 nM TaqMan hybridization probe were used in a 25 μl volume. The probe of real-time PCR was labeled with carboxylfluorescein (FAM) at the 5' end and with the quencher carboxytetramethylrhodamine (TAMRA) at the 3' end. The MUC8 and β2-microglobulin primers and TaqMan probe used are described in Table 3.

Product	Primer	Oligonucleotide Sequence
MUC8	Forward	5'-TAACCCAATGCCACTCCTTC-3'
	Reverse	5'-GGAGTGTAACCTGGCTGCTC-3'
	Taqman probe	6FAM-GGTTAGGGCTGACCACAGAA-TAMRA
β2-M	Forward	5'-CGCTCCGTGGCCTTAGC-3'
	Reverse	5'-GAGTACGCTGGATAGCCTCCA-3'
	Taqman probe	6FAM-TGCTCGCGCGCTACTCTCTCTTTCTGGC-TAMRA

Table 3. Experimental primer and Taqman oligonucleotide sequences used in real-time PCR.

Real-time reverse transcription-PCR was performed on a PE Biosystems ABI PRISM® 7700 sequence detection system (Foster City, CA). The thermocycler

parameters were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reaction was performed in triplicate. Relative quantity of *MUC8* mRNA was obtained using a comparative threshold method, and results were normalized against ß2-microglobulin as an endogenous control.

Western Blot analysis

NHNE cells were grown to confluence in 6-well plates. After treating with 25ng/ml, PDGF for 5, 10, 30, 60, or 120 min, the cells were lysed with 2X lysis buffer (250 mM Tris-Cl (pH6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% bromphenol blue, 10% glycerol). Equal amounts of whole cell lysates were resolved on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane in Tris-buffered saline (TBS; 50 mMTris-Cl (pH 7.5), 150 mM NaCl) for 1 h at room temperature. The blot was incubated overnight with primary antibody in TTBS (0.5% Tween 20 in TBS). After washing with TTBS, the blot was incubated for 1 h at room temperature with anti-rabbit or anti-mouse antibody (Cell signaling) in TTBS and visualized using the ECL system (Amersham, Little Chalfont, UK).

Intracellular ROS Assay

After stimulating confluent cells with PDGF (25 ng/ml) for 10, 20, 30, and 60 min, they were washed with RPMI (lacking phenol red) and incubated for 10 min in the dark in Krebs-Ringer solution containing 5 μM 2',7'-dichlorofluorescin diacetate (DCF-DA). DCF-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the

non-fluorescent polar derivative DCF and trapped within the cells 24. We washed the cells with 1 ml of HBSS at least five times to get rid of exogenous H_2O_2. In the presence of a proper oxidant, this compound is converted into 2'7'-dichlorofluorescin (DCF) by intracellular esterases and then oxidized to the highly fluorescent 2'7'-DCF by ROS or reactive nitrogen species (RNS). Culture dishes were viewed on a Zeiss Axiovert 135 inverted confocal microscope equipped with an x20 Neofluor objective and Zeiss LSM 410 confocal attachment. DCF fluorescence was measured at an excitation wavelength of 488nm and emission at 515-540nm. Seven fields of each dish were randomly selected and the fluorescence intensity was measured with the Karl Zeiss vision system (KS400, version 3.0). The seven values were averaged to obtain the mean relative fluorescence intensity, and the mean relative fluorescence intensities were used for comparisons. All experiments were repeated at least three times.

Cell transfection with Nox2 and Nox4 siRNA

Specific siRNA against Nox2 and Nox4 was used to suppress their respective expressions. The transfection rates of Nox2 or Nox4 siRNA were verified to be over 90% in NCI-H292 cells. NCI-292 cells were transiently transfected with Nox2, Nox4 siRNA using oligofectamine™ reagent following the manufacturer's instruction (Invitrogen). 1ug of each siRNA and 1λ oligofectamine™ were mixed with RPMI without serum and antibiotics respectively and then transfection was performed onto 6-well NCI-H292 cell plates. This procedure did not affect cell viability and after 48 hrs of transfection and cellular deprivation was performed according to usual

methods. The same procedure was performed with **c**ontrol siRNA (200 nmol/l, Dharmacon)**.**

Adenoviral transduction of human airway epithelial cells.

Control and recombinant adenoviruses encoding Nox4 construct were incubated with NCI-H292 cells for 60 min at room temperature in RPMI. After incubation, adenovirus infection of NCI-H292 cells was performed by overnight incubation in RPMI containing 10% fetal bovine serum and penicillin/streptomycin. The next day, the medium was replaced with complete RPMI containing 10% fetal bovine serum and penicillin/streptomycin. The cells were used for experiments 48 hr posttransduction after they were starved for 24 hr in RPMI medium containg 0.5% fetal bovine serum.

Statistical analysis

At least three separate experiments were performed for each measurement and the data are expressed as mean $\pm$ standard deviation (SD) of triplicate cultures. Differences between treatment groups were assessed by analysis of variance (ANOVA) with a *post hoc* test. Differences were considered statistically significant at $p < 0.05$.

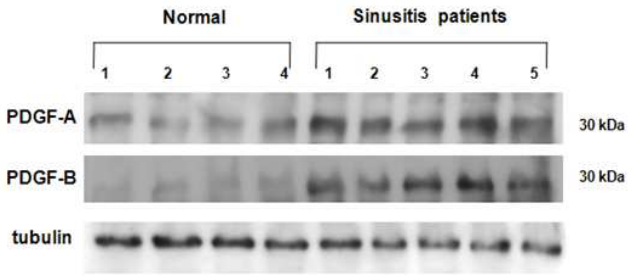
III. Results

The expression of PDGF-A and B protein is higher in the tissue of sinusitis patients, comparing the normal tissue.

We obtained the normal tissue and nasal polyp from the 9 volunteers, including 4 volunteers having normal sinus and the other 5 with chronic sinusitis, to examine whether PDGF level is increased in chronic infection of paranasal sinus. We performed western blot analysis using polyclonal PDGF-A and B antibody and the result showed that the expression of PDGF-A was increased in the patients with sinusitis (4.2 fold over normal), comparing the expression in the normal subjects. Similar pattern of results was shown in the expression of PDGF-B (over 10.1 fold over normal, Figure 1A, 1B).

Figure 1

(A)



(B)

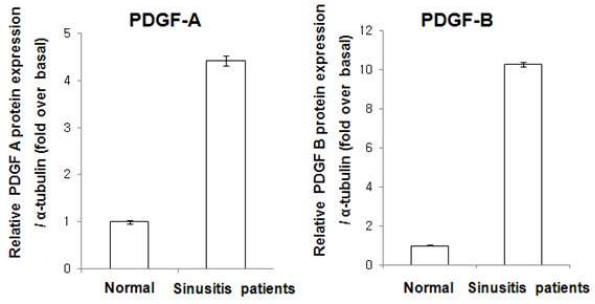


Fig. 1. (A) Western blot analysis for PDGF-A and B in the tissues of normal volunteers and sinusitis patients. Western blot analysis demonstrating the protein expression of PDGF-A and PDGF-B was increased in the tissue of sinusitis patients (N=5), comparing to the expression in the tissue of normal volunteers (N=4). (B) Densitometry for the expression of PDGF-A and B. The amount of PDGF increased in the tissue of sinusitis patients. The results of densitometry are presented as mean ± standard deviation (SD).

These findings demonstrate that the expression of PDGF-A and B is increased in the sinusitis patients than normal and the increased-level of PDGF may play a role in the pathogenesis of chronic sinusitis.

PDGF induces *MUC8* gene expression in a dose and time-dependent manner, but not *MUC5AC, MUC1* and *MUC4* expression.

To examine which mucin genes could be induced by stimulation of PDGF, RT-PCR was performed after treating cells (1 x 10^6/ml) with PDGF-BB (5, 10, 25, 50, and 100 ng/ml) for 24 h. *MUC8* mRNA levels increased after PDGF treatment in a dose-dependent manner, but *MUC5AC, MUC1, and MUC4* mRNA levels did not (Figure 2A). This result shows that among membranous mucin genes, PDGF specifically induces *MUC8* gene expression and comparing secreted mucin genes, PDGF does not affect to the gene expression of *MUC5AC*, major secreted mucin gene. Real-time PCR revealed that *MUC8* gene expression was significantly higher after treatment with 25 ng/ml (3.48±0.38 fold over control; $p < 0.05$), 50 ng/ml (4.30±0.23 fold over control; $p<0.05$) and 100 ng/ml PDGF (4.98±0.30 fold over control; $p<0.05$)

(Figure 2B). After PDGF treatment for 2, 4, 8, 12, and 24 hrs, we carried out real-time PCR. *MUC8* gene expression was higher starting from 8 hr after treatment (8 hr: 3.45±0.98, 12 hr: 4.06±0.32, 24 hr: 4.61±0.74 fold over control; $p < 0.05$; Figure 2C). We used 25 ng/ml PDGF for all subsequent experiments.

Fig. 2.

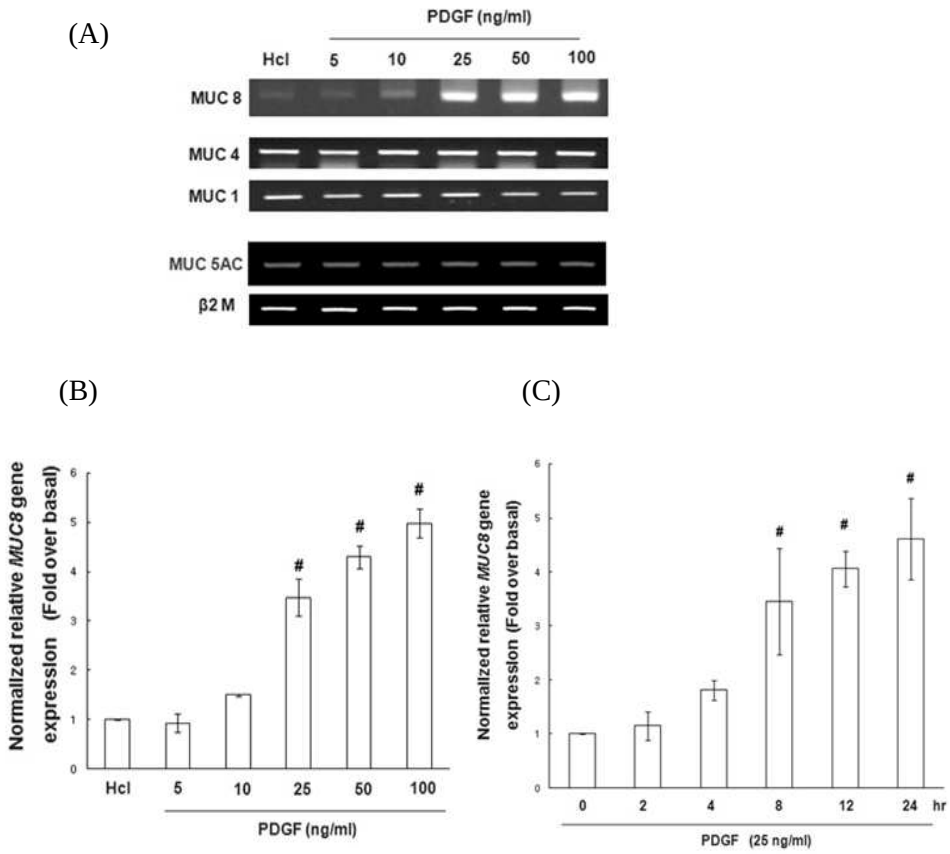


Fig. 2. (A) RT-PCR for MUC8, MUC4, MUC1, MUC5AC. NHNE cells were treated with PDGF (5 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml) for 24 hr. β2-microglobulin (β2M) was used as an internal control. RT-PCR showing PDGF induced *MUC8* gene expression as dose-dependent manners. (B) Real-time PCR demonstrating the dose-dependent effect of PDGF

on *MUC8* gene expression after 24 hr. (C) Real-time PCR demonstrating the time-dependent effect of PDGF (25 ng/ml) on *MUC8* gene expression. The results are presented as mean ± SD of triplicate cultures (#:$p < 0.05$ when compared with control).

PDGF induces intracellular ROS generation through NADPH oxidase.

NHNE cells were stimulated with media containing 25 ng/ml PDGF for 10, 20, 30 and 60 min. The amount of ROS was measured using a fluorescence-based assay with 2',7'- DCFH-DA and laser-scanning confocal microscopy. The stimulation of NHNE cells with PDGF resulted in a time-dependent increase in the intensity of DCF fluorescence, with the maximal increase (4.64-fold) apparent 10 min after stimulation and fluorescence had diminished by 60 min (Figure 3A). We next examined whether increased amount of intracellular ROS was involved in PDGF-induced *MUC8* gene overexpression. NHNE cells were treated with 10μM NAC, ROS scavenger 1 hr before the stimulation with PDGF (25 ng/ml) and performed intracellular ROS assay and real-time PCR. Pretreatment with NAC suppressed PDGF-induced intracellular ROS generation. In addition, increased level of *MUC8* gene expression was suppressed significantly in case of pretreatment with NAC (4.01±0.40 versus 1.38±0.39 fold over control; $p<0.05$) (Figure 3B).

Fig. 3.

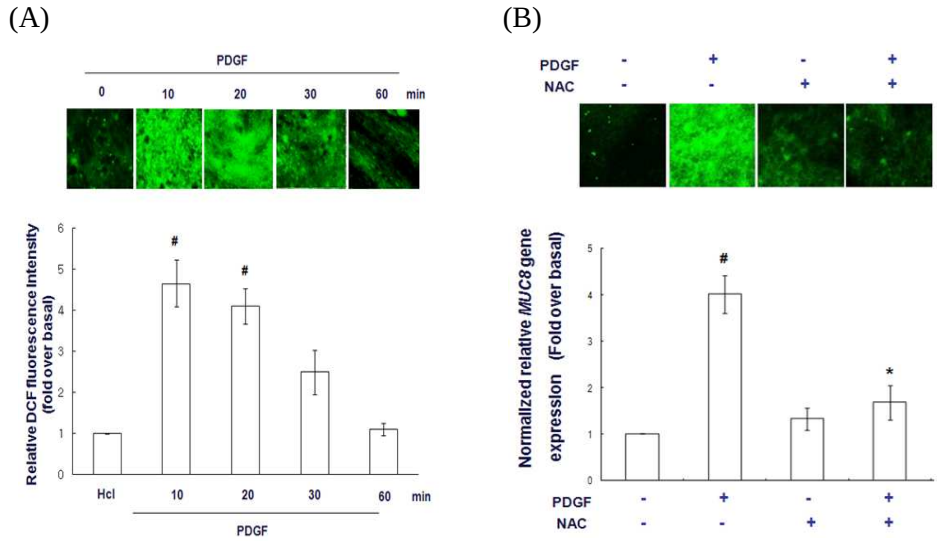


Fig. 3. (A) Intracellular ROS assay after treatment with PDGF. NHNE cells were stimulated with media containing 25 ng/ml PDGF for 10, 20, 30, or 60 min, and the production of intracellular ROS was measured using a fluorescence-based assay with 2', 7'-DCF-DA and laser-scanning confocal microscopy. The figures of fluorescent intensity are representative of three separate experiments, and the graph of intensity depicts mean ± SD of triplicate cultures (#:$p < 0.05$). (B) Intracellular ROS assay and real-time PCR after pretreatment with NAC. After pretreatment with NAC (30 uM), we measured the change of intracellular ROS. Intracellular ROS assay and real-time PCR shows the effect of NAC on PDGF (25 ng/ml)-induced intracellular ROS generation and *MUC8* gene expression. The results are presented as mean ± SD of triplicate cultures (#:$p < 0.05$ when compared with control, *:$p < 0.05$ when compared with PDGF treatment group).

These data suggest that PDGF can produce intracellular ROS and increased amount of intracellular ROS promoted *MUC8* gene overexpression in NHNE cells. To determine which enzyme is involved in PDGF–induced intracellular

ROS generation, we inhibited each enzymes, involved in intracellular ROS generation, using Diphenyleneiodium chloride (DPI, Nox inhibitor), N^G-Monoethyl-L-arginine (NMEA. NO synthase inhibitor), allopurinol (xanthine oxidase inhibitor) and dicumarol (NADPH:quinine oxidoreductase inhibitor). After pretreating NHNE cells with DPI 30 uM, NMEA 10 uM, allopurinol 100 uM or dicumarol 30 uM, we measured the change of intracellular ROS and performed real-time PCR to examine *MUC8* gene expression. NMEA, allopurinol and dicumarol did not have a significant inhibitory effect on PDGF-induced intracellular ROS or *MUC8* gene overexpression. In contrast, pretreatment with DPI, suppressed PDGF-induced intracellular ROS and *MUC8* gene expression (4.72±0.23 versus 1.82±0.06 fold over control; $p < 0.05$) (Figure 4).

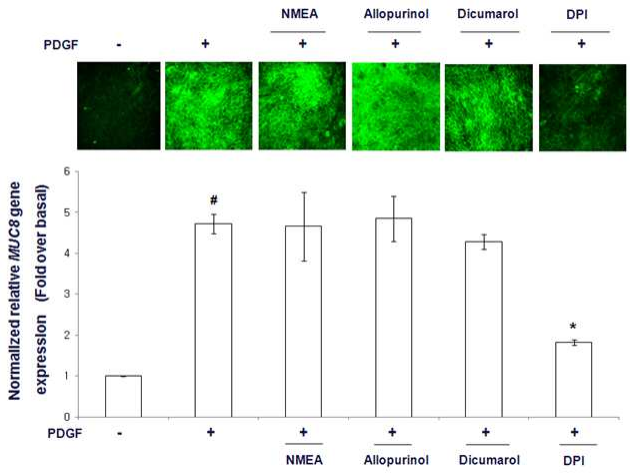


Fig. 4. Intracellular ROS assay and real-time PCR after inhibition of enzymes to generate intracellular ROS. After pretreatment with NMEA (10 uM), allopurinol (100 uM), dicumarol (30 uM), and DPI (30 uM), we measured the change of intracellular ROS and *MUC8* gene expression. Intracellular ROS assay and real-time PCR shows the effect of each inhibitor on

PDGF (25 ng/ml)-induced intracellular ROS production and *MUC8* gene expression. The results are presented as mean ± SD of triplicate cultures (#:$p < 0.05$ when compared with control, *:$p < 0.05$ when compared with PDGF treatment group).

These results suggest that PDGF can produce intracellular ROS through Nox and the activation of Nox may affect *MUC8* gene overexpression through the generation of intracellular ROS.

Nox4 is the primary Nox homolog involved in PDGF-induced intracellular ROS generation and *MUC8* gene overexpression in NHNE cells.

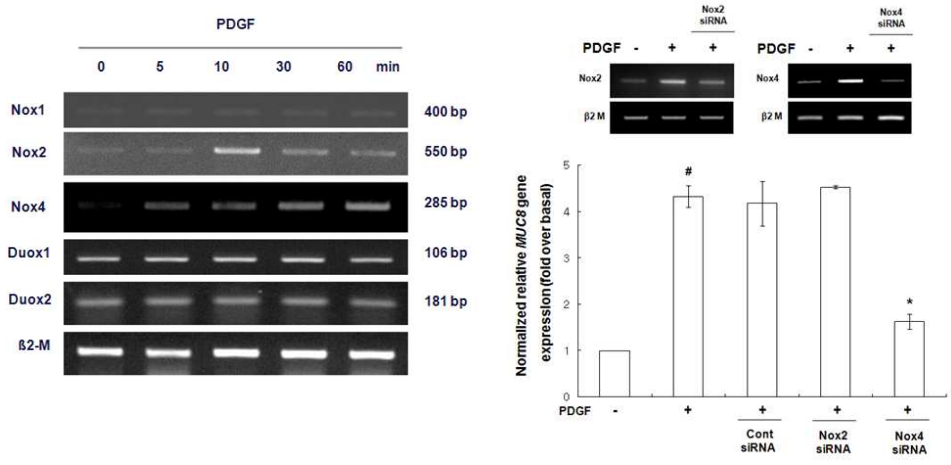
To determine whether PDGF promotes a significant increase in the expression of Nox subtypes and to identify which Nox subtypes may be involved in intracellular ROS generation within NHNE cells, cells were treated with PDGF in a time-dependent manner and RT-PCR for Nox subtypes was performed. The PCR finding showed that *Nox4* and *Nox2* gene expressions increased significantly 5, 10 min respectively after stimulation with PDGF (25 ng/ml). Expression of *Nox1, Duox1*, and *Duox2* genes did not increase and, *Nox3* and *Nox5* were not expressed in NHNE cells after stimulation with PDGF (Figure 5A). Having established the role of Nox2 and Nox4 in *MUC8* gene overexpression by PDGF-induced intracellular ROS, we subjected the cells to transient transfection with siRNA for Nox2 and Nox4. We obtained the same results when the experiments (Figure 3A, 3B, 4, 5A) were performed using NCI-H292 cells. Accordingly, we subjected NCI-H292 cells to transient transfection and infection for Nox subtypes to verify the

critical function of Nox in PDGF-induced intracellular ROS generation.

After transfection with Nox4 siRNA, PDGF-induced *MUC8* gene overexpression was suppressed (4.33±0.22 versus 1.63±0.16 fold over control; $p < 0.05$) but transfection with Nox2 siRNA did not reduce PDGF-induced *MUC8* gene overexpression comparing with control siRNA (4.33±0.22 versus 4.53±0.05 fold over control; $p < 0.05$) (Figure 5B). We next performed western blot analysis to examine whether Nox4 protein is activated after stimulation with PDGF in NHNE cells. Western blot analysis demonstrated that the expression of Nox4 protein peaked at 5 min after stimulation with PDGF (25 ng/ml) (Figure 5C).

Fig. 5

(A) (B)



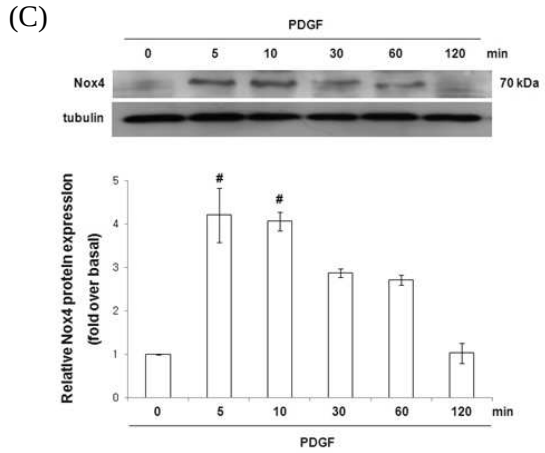


Fig. 5. (A) RT-PCR for Nox subfamily. NHNE cells were stimulated with PDGF (25 ng/ml) for 5, 10, 30, or 60 min. RT-PCR shows that *Nox4* and *Nox2* gene expression increased significantly 5 and 10 min after stimulation with PDGF. (B) Real-time PCR after transfection with Nox2 and Nox4 siRNA. Real-time PCR showing the effect of inhibition of Nox2 and Nox4 with each siRNA on PDGF-induced *MUC8* gene expression. (C) Western blot analysis for Nox4 after stimulation with PDGF. NHNE cells were stimulated with PDGF (25 ng/ml) for 5, 10, 30, 60, or 120 min. Western blot analysis demonstrating the maximum increase of Nox4 protein was observed 5, 10 min after stimulation with PDGF. The results of western blot analysis, RT-PCR and real-time PCR are representative of three separate experiments, and the results of densitometry are presented as mean $\pm$ SD of triplicate cultures (#:$p < 0.05$ when compared with control, *:$p < 0.05$ when compared with PDGF treatment group).

The regulation of Nox4 is responsible for change in PDGF-induced intracellular ROS generation and *MUC8* gene expression.

To study the specific function of Nox4 in PDGF-induced intracellular ROS, resulting in *MUC8* gene overexpression, NCI-H292 cells were infected with

adenovirus encoding Nox4 (AdNox4), in addition to transfection with Nox4 siRNA. Transfection with Nox4 siRNA specifically reduced *Nox4* gene expression (Figure 6A) and resulted in decreasing PDGF-induced *MUC8* gene expression (4.77±0.09 versus 1.70±0.06 fold over control; $p < 0.05$) and intracellular ROS (4.37±0.40 versus 1.47±0.28 fold over control; $p < 0.05$), compared with transfection using control siRNA (Figure 6B, 6C).

Infection with AdNox4 resulted in significant increased *Nox4* gene expression (Figure 6A) and induced *MUC8* gene expression with treatment of PDGF (6.29±1.27 versus 1.72±0.49 fold over control; $p < 0.05$) and intracellular ROS (6.78±1.38 versus 4.22±0.20 fold over control), compared with infection using control adenovirus. In case of only infection with AdNox4 without PDGF treatment, real-time PCR showed that *MUC8* gene expression was higher, comparing with cells which is infected control adenovirus (3.11±0.37 versus 1.72±0.43 fold over control). These findings demonstrated that Nox4 is a critical Nox homolog to produce intracellular ROS in NHNE cells and is essential for PDGF-induced intracellular ROS generation and *MUC8* gene expression.

Fig. 6.

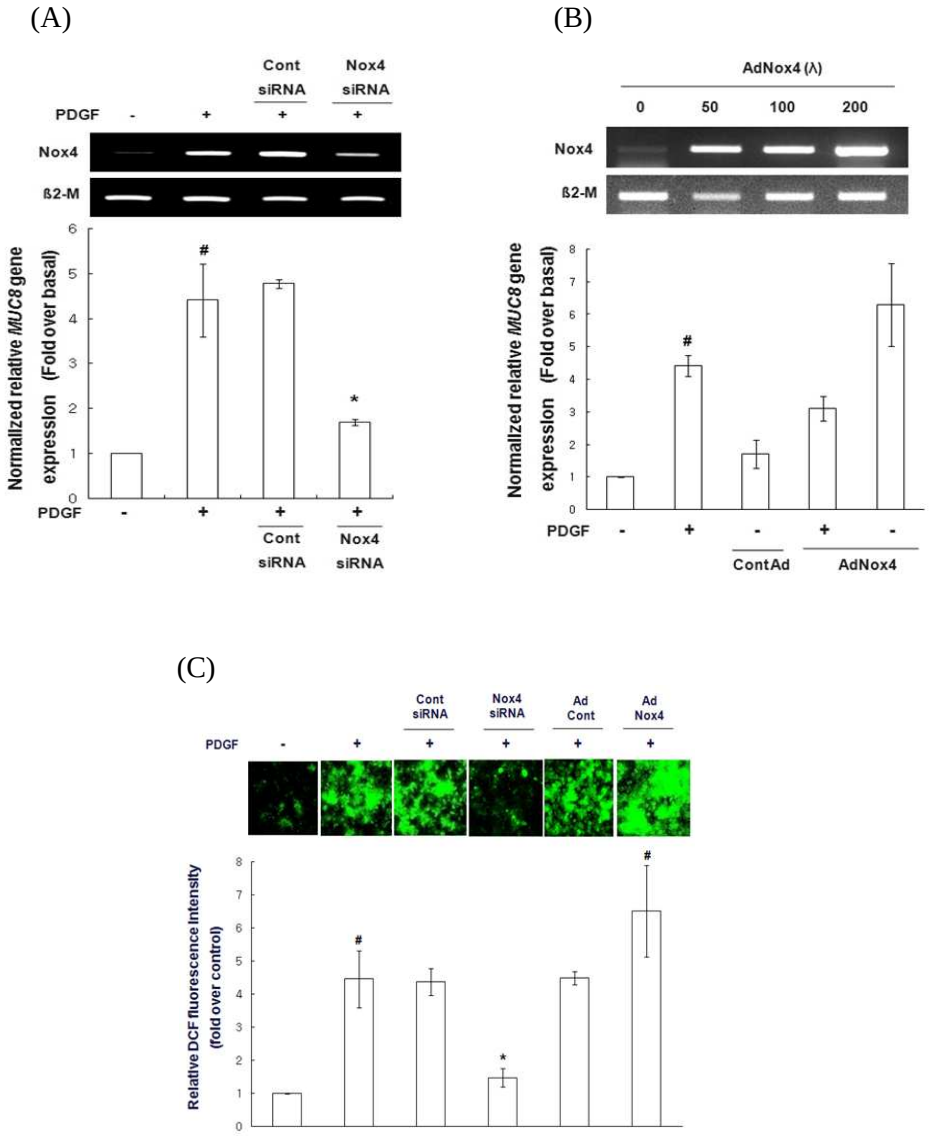


Fig. 6. (A) The increased Nox4 gene expression was suppressed after transfection with Nox4 siRNA. Real time-PCR shows that *MUC8* expression was suppressed after transfection with Nox4 siRNA. (B) *Nox4* gene expression was increased after infection with Nox4 overexpression vector (AdNox4). Real-time PCR shows that *MUC8* gene expression was increased after infection with AdNox4, independently stimulation with PDGF. The results are

presented as mean ± SD of triplicate cultures (#:p < 0.05 when compared with control, *:p < 0.05 when compared with PDGF treatment group). (C) Intracellular ROS assay after transfection with Nox4 siRNA and infection with Nox4 overexpression vector. Intracellular ROS assay demonstrating the effect of Nox4 siRNA and AdNox4 on PDGF-induced intracellular ROS production. The figures of fluorescent intensity are representative of three separate experiments, and the graph of intensity depicts mean ± SD of triplicate cultures (#:p < 0.05 when compared with control, *: p < 0.05 when compared with PDGF treatment group).

We also carried out western blot analysis for Nox4 protein expression, using human tissue of normal volunteer and chronic sinusitis patients. The expression of Nox4 protein was increased in the tissue of patients with chronic sinusitis (over 8.2 fold), comparing the expression in that of the normal volunteer (Figure 7).

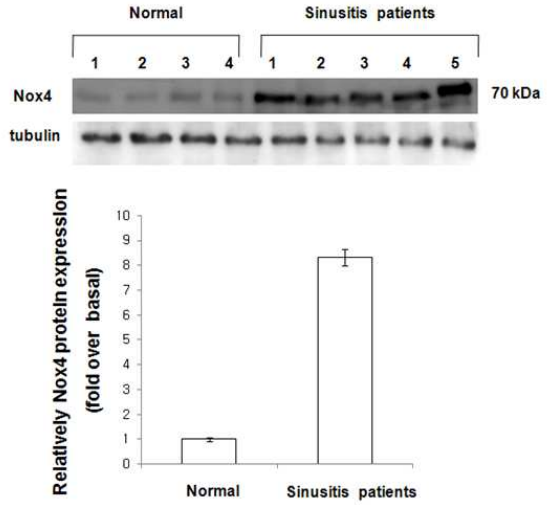


Fig. 7. Western blot analysis for Nox4 in the tissues of normal volunteers and sinusitis patients. The amount of Nox4 increased in the tissue of sinusitis patients. Western blot analysis demonstrating the protein expression of Nox4 was increased in the tissue of sinusitis

patients (N=5), comparing to the expression in the tissue of normal volunteers (N=4). The results of densitometry are presented as mean ± SD.

Importantly, these findings showed that Nox4 can be expressed more abundantly in nasal tissue with chronic sinusitis than normal nasal tissue and suggests it may be involved in the pathogenesis of mucin gene expression and chronic infection of nasal tissue through intracellular ROS generation.

Phosphorylation of ERK1/2 MAP kinase is involved in PDGF-induced intracellular ROS generation and *MUC8* gene expression.

As a next step, we examined the involvement of the MAP kinase signal pathway as a upstream signal of Nox4. ERK1/2 MAP kinase was maximally activated after 5, 10 min of stimulation with PDGF (3.92±0.32, 4.09±0.66, fold over control; $p < 0.05$) and gradually decreased thereafter in NHNE cells (Figure 8A). No significant change was detected in p38 and JNK activation (data not shown). In order to investigate the relations between PDGF receptor and ERK1/2 MAP kinase in PDGF-induced *MUC8* gene expression, we first pretreated NHNE cells with STI571(1 uM), a PDGF receptor inhibitor, for 1 hr before treatment with PDGF. western blot analysis and real time-PCR clearly showed that STI571 pretreatment inhibited the phosphorylation of ERK1/2 MAP kinase and PDGF-induced *MUC8* gene expression (4.12±0.42

versus 1.81±0.19 fold over control; p <0.05; Figure 8B).

Fig. 8.

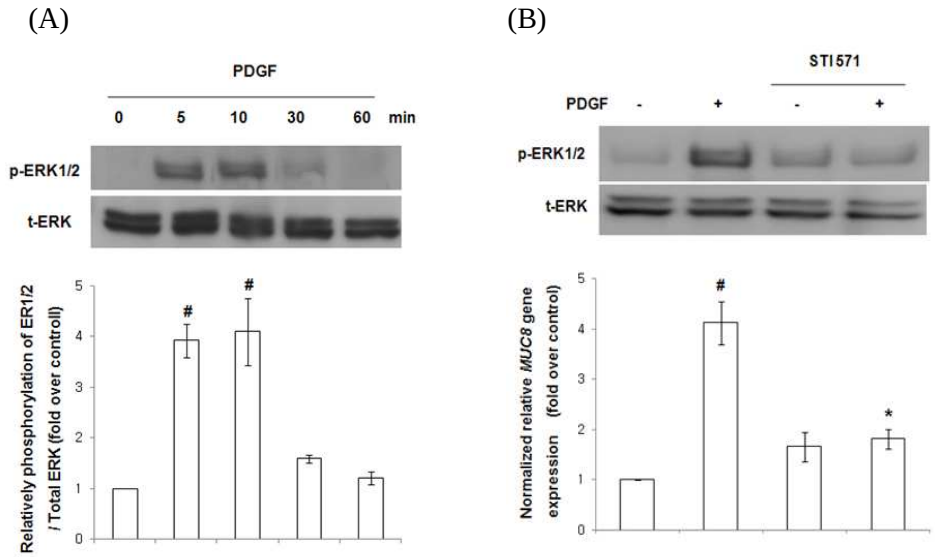


Fig. 8A. Effect of PDGF on the activation ERK1/2 MAP kinase. NHNE cells were treated with PDGF (25 ng/ml) for 5, 10, 30, or 60 min. Western blot analysis demonstrates the effect of PDGF on ERK1/2 MAP kinase. (B) Effect of PDGF on the activation ERK1/2 MAP kinase. NHNE cells were treated with STI571 (gleevec, 1 uM) for 1 hr before treatment with PDGF (25 ng/ml). Western blot analysis and real-time PCR show that pretreatment with STI571 inhibited the activation of ERK1/2 MAP kinase and PDGF-induced *MUC8* gene overexpression. The result of western blot analysis is representative of three separate experiments, and the results are presented as mean ± SD of triplicate cultures (#:p < 0.05 when compared with control, *:p < 0.05 when compared with PDGF treatment group).

To study the specificity of ERK1/2 MAP kinase, cells were transfected

transiently with ERK1 or ERK2 MAP kinase siRNA, respectively. Interestingly, transfection with ERK1 and ERK2 MAP kinase siRNA specifically reduced PDGF-induced Nox4 protein expression, intracellular ROS generation, and *MUC8* gene expression compared with control siRNA transfection (sc-37007) (4.55±0.25 versus 1.40±0.02, 1.67±0.10 fold over control; $p < 0.05$, Figure 9A, 9B).

Fig. 9

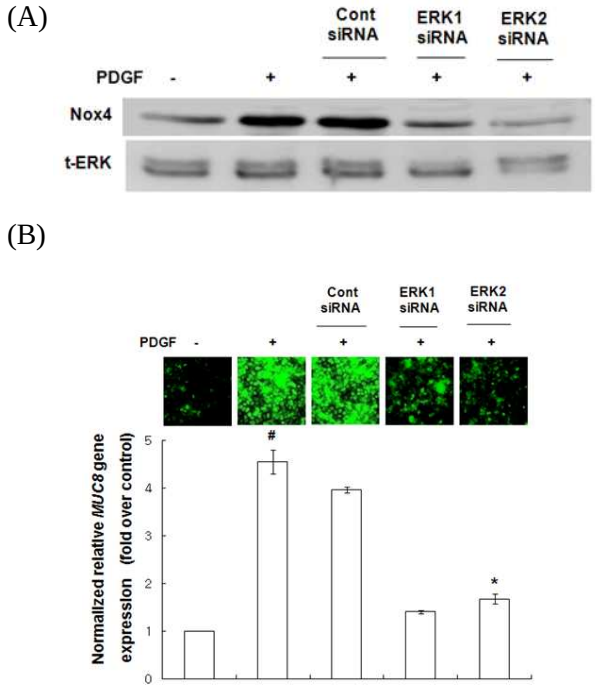


Fig. 9. (A) Nox4 produced intracellular ROS through the activation of ERK1/2 MAP kinase. NCI-H292 cells were transfected with ERK1 and ERK2 MAP kinase siRNA. Western blot analysis shows that Nox4 protein expression decreased after transfection with ERK1 and ERK2 siRNA, respectively. (B) Nox4 produced intracellular ROS through the activation of ERK1/2

MAP kinase. Intracellular ROS assay and real-time PCR demonstrates that transfection with ERK1 and ERK2 siRNA inhibited PDGF-induced intracellular ROS generation and *MUC8* gene expression. The results of western blot analyses and intracellular ROS assay are representative of three separate experiments, and the results are presented as mean $\pm$ SD of triplicate cultures (#:$p < 0.05$ when compared with control, *:$p < 0.05$ when compared with PDGF treatment group).

These results indicate that PDGF-induced *MUC8* gene expression requires the activation of ERK1/2 MAP kinase and subsequent activation of Nox4 in NHNE cells.

IV. Discussion

In this study, we presented that in chronic sinusitis, PDGF expression was increased and stimulated intracellular ROS generation in NHNE cells, resulting in *MUC8* gene overexpression. We have focused on the role of Nox4 in generating intracellular ROS required for *MUC8* gene expression.

The airway epithelium is constantly exposed to oxidant generated internally and is vulnerable to oxidant damage. ROS generation in response to variety stimuli is considered as an essential pathologic effect to the host and the effect on host tissue may manifest as airway inflammatory, allergic, or autoimmune diseases[26-29]. Many growth factors can mediate intracellular

signaling, such as transforming growth factor ß1, basic fibroblast growth factor, epidermal growth factor (EGF), vascular endothelial growth factor and PDGF[30-33] and especially, PDGF plays an important role in cell proliferation, differentiation and chemotaxis and involved in various disorders[19,34]. In our study, PDGF expression is increased significantly in the tissue of patients with chronic sinusitis, comparing to the normal tissue. We also found that PDGF can increase *MUC8* gene expression, known as an important mucin gene in chronic sinusitis and nasal polyp[10]. From these findings, we suggest that PDGF can promote the pathogenesis of chronic infection through *MUC8* gene overexpression in NHNE cells.

ROS has been known to be closely related with airway inflammation and increased oxygen burden may arise harmful effect to respiratory tract[29]. Physiologically, ROS induce remodeling of extracellular matrix and blood vessel, stimulate mucus secretion and alveolar repair response[27]. PDGF can use and produce ROS as a mediator for intracellular signaling in various cell types, such as HepG2 cells, hepatic stellate cells, pulmonary artery smooth muscle cells and fibroblasts[35-38]. Our results showed that PDGF produced intracellular ROS in NHNE cells and scavenging of ROS by NAC completely inhibited PDGF-induced *MUC8* gene overexpression. Therefore, PDGF can

induce chronic infection through *MUC8* gene overexpression in NHNE cells and intracellular ROS is an important mediator in PDGF-induced *MUC8* gene overexpression.

It has been presented that ROS is an essential substrate for inflammation, tumorigenesis and apoptosis. Accordingly, the study to identify the enzymatic source of ROS is important in clinical practice. Nox plays a key role in ROS generation in response to a number of stimulants[6,38,39]. In this study, we presented that in NHNE cells, Nox is unique source in PDGF-stimulated intracellular ROS generation. The activity of Nox is significantly increased by various specific stimuli and Nox homolog expression has diverse cell-specific associations[40,41]. It has been suggested that Duox is the major Nox homolog found in airway epithelium[11,14,42-44]. Duox1 can be activated by PMA or neutrophil elastase, producing ROS and resulting in MUC5AC mucin overproduction in human bronchial epithelial cells[11]. In addition, Duox1 and Duox2 gene and protein were expressed dominantly in human trachea, localized in the apical portion of epithelial cells[14]. Our previous study have suggested that exogenous H_2O_2 increases the expression of Nox4 in NHNE cells and Nox4 is expressed predominantly in goblet cell of airway epithelial cells[15]. We performed real-time PCR for 7 Nox homologs with human

turbinate tissue in nasal cavity and among them, Nox2 and Nox4 were highly expressed (data was not shown). In the present study, we found that gene expression of Nox2 and Nox4 were higher by treatment with PDGF in NHNE cells, along with the increase of intracellular ROS generation. Expressions of Nox1, Duox1, and Duox2 were unchanged and expression of Nox3 and Nox5 were not detected in NHNE cells. However, specific inhibition of Nox4 decreases PDGF-induced intracellular ROS generation and *MUC8* gene overexpression, whereas inhibition of Nox2 does not. Knocking-down experiments demonstrated that Nox4 has a critical role in PDGF-inducing *MUC8* gene overexpression through intracellular ROS generation.

It has been known that Nox4 expression is strongly observed in the kidney, placenta, pancreas and endothelial cells[45,46]. In addition, several evidences indicated that Nox4 plays an important role in intracellular ROS generation in vascular endothelial cells[2], osteoclast[28] skin fibroblast[38] and adipocyte[39]. Our study showed that Nox4 can produce intracellular ROS and induce *MUC8* gene expression in response to PDGF stimulation in NHNE cells. Down-regulation of Nox4 contributes to the impaired ROS generation and decreased *MUC8* gene expression. Moreover, overexpression of Nox4 can

increase intracellular ROS generation leading to *MUC8* gene overexpression, regardless of PDGF in NHNE cells. These findings demonstrates that Nox4 is a critical enzymatic source of ROS generation in NHNE cells and Nox4-generated ROS is a mediator of *MUC8* gene overexpression in response to PDGF stimulation.

The signal pathways behind the response to growth factors and the generation of intracellular ROS have not been fully evaluated in airway epithelial cells. However, MAP kinase signal pathway has been reported to be related with cell differentiation and proliferation in airway epithelial cells. We observed that stimulation with PDGF promoted the generation of intracellular ROS through the activation of Nox4, and ERK1/2 MAP kinase signal transduction mediated the Nox4-induced intracellular ROS generation in NHNE cells. In other words, PDGF-induced *MUC8* gene expression may be associated with intracellular ROS generation through ERK1/2 MAP kinase signal pathway in NHNE cells.

PDGF-induced intracellular ROS generation and Nox4 expression start at 10 min after stimulation. However, *MUC8* gene expression increased 8 hr after stimulation with PDGF. We thought that this time difference may be originated from histopathologic changes of airway epithelium. The amount of

PDGF is increased in inflammation in normal nasal epithelium and it can induce the gene and protein expression of Nox4 immediately. Nox4 starts to produce intracellular ROS and these intracellular ROS generate histopathologic change in nasal epithelium, such as goblet cell hyperplasia and submucous glandular change. We suggested that intracellular ROS-induced histopathologic change may take at least 8 hrs in nasal epithelium and finally, it induces mucin gene transcription and expression.

V. Conclusion

PDGF-A and B expressed higher in the patients with chronic sinusitis. This increased PDGF induces intracellular ROS generation, resulting in *MUC8* gene expression in NHNE cells. The expression of Nox4 is also increased in sinusitis patients and in NHNE cells response to PDGF. In present study, we thought that PDGF and Nox4 play an essential role in pathologic mucin gene expression and may be correlated with the mechanism of chronic sinusitis through intracellular ROS generation.

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Abstract (in korean)

사람 기도 상피세포에서 혈소판 유래 성장 인자에 MUC8 과발현의 신호 전달 기전

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김 현 직

활성 산소가 다양한 염증성 기도 질환의 병인에 중요한 역할을 함과 NADPH oxidase 같은, 특정 효소들이 이러한 활성 산소를 세포 내에서 생성한 다는 것은 이미 잘 알려 져 있다. 혈소판 유래 성장인자, 내피성 성장인자 같은 성장 인자들은 세포 내 활성 산소의 생성을 유도하고 만성 염증성 기도 질환의 주 증상인 점액 과분비를 일으킬 수 있다.

본 연구의 목적은 사람정상코상피세포에서 혈소판 유래 성장인자의 자극에 의해 어떤 점액 유전자의 발현이 증가되는 지 알아 보고 이러한 유전자가 증가하는 기전에 대해 연구하고자 시행되었다.

혈소판 유래 성장 인자의 발현은 정상 조직 보다 부비동염이 있는 환자의 조직에서 더 증가됨을 알 수 있었고 세포 내 활성산소의 생성을 통해 MUC8유전자의 발현을 증가시킴을 알 수 있었다.

NADPH oxidase의 subfamily중 하나인 Nox4가 사람정상코상피세포에서 이러한 활성 산소의 생성과 혈소판 유래 성장 인자에 의해 유도 된 MUC8 의 발현 증가에 중요한 역할을 하며 세포 신호전달체계 중 ERK1/2 MAP kinase가 PDGF의 자극에 의해 Nox4를 활성화 시키는 과정에서 매개체 역할을 할 것으로 생각된다.

핵심 되는 말 : PDGF, MUC8, Nox4, ROS