The Critical role of Nox4 in oxidative stress-induced *MUC5AC* overexpression in normal human nasal epithelial cells

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Ju hyun Jeon

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This certifies that the Master's Thesis
of Ju hyun Jeon is approved.

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The Graduate School
Yonsei University

June 2008

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The Critical role of Nox4 in oxidative stress-induced *MUC5AC* overexpression in normal human nasal epithelial cells

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Mucus hypersecretion is a prominent manifestation in patients with chronic inflammatory airway diseases, and MUC5AC is a major airway mucin. It is well known that reactive oxygen species (ROS) may be involved in the pathogenesis of various inflammatory airway diseases. The purpose of this study was to identify which secreted mucin genes are induced by exogenous hydrogen peroxide (H_2O_2) and the mechanism by which these genes are up-regulated in normal human nasal epithelial (NHNE) cells. Exogenous H_2O_2 induced the ligand-independent activation of epidermal growth factor receptors (EGFR) and subsequent activation of ERK1 MAP kinase, resulting in induction of intracellular H_2O_2 generation. Through this signal pathway, exogenous H_2O_2 markedly induced overexpression of only *MUC5AC* gene. In addition, Nox4, a subtype of non-phagocytic NADPH oxidase, was found to play a key role in intracellular H_2O_2 generation and exogenous H_2O_2–induced *MUC5AC* gene expression in NHNE cells.

Key words: hydrogen peroxide; *MUC5AC*; EGFR; Nox4

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I. INTRODUCTION

Mucin hypersecretion is commonly observed in respiratory diseases such as rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis and cystic fibrosis [1-3]. To date, 20 different mucin genes have been identified and subdivided into two groups, the membrane-bound and secreted mucins. *MUC5AC, MUC5B, MUC6, MUC7 and MUC19* are the secreted mucins [4-8]. *MUC5AC* and *5B* are the major secreted mucins, highly expressed in the goblet cells of human airway epithelium and the submucosal glands [9-11].

Oxidative injury triggered by either inhaled or locally generated reactive oxygen species (ROS), elicits an inflammatory response that can profoundly impair the structural integrity and biological properties of bronchial epithelium [3, 9-12]. There

are several potential sources of ROS in most cells, including nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, xanthine oxidase, uncoupled nitric oxide (NO) synthase and the mitochondrial respiratory chain reaction. Exogenously, ROS can be produced in response to a variety of extracellular stimuli such as air pollutants or cigarette smoking. A large number of studies have demonstrated that ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2-), and hydroxyl radical, play a role in the progression of many airway diseases and mucin gene expression in human airway epithelial cells [13, 14]. The mechanism of H_2O_2 generation has been studied extensively in phagocytic cells, in which O_2^- is produced via the one-electron reduction of O_2 by the multicomponent NADPH oxidase (Nox) system [15]. In contrast, the mechanism behind H_2O_2 generation in non-phagocytic cells remains unclear but evidence suggests that the system responsible for H_2O_2 generation in non-phagocytic cells is structurally and genetically distinct from, but functionally similar to, the Nox system of phagocytes [16, 17]. To date, 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). Recently, Duox1 was identified in normal human bronchial epithelial cells and shown to generate ROS [18, 19]. Nox4 is highly expressed in endothelial cells [20] but there have been few reports about its expression in airway epithelium.

The regulation of gene expression by oxidative stress involves numerous signaling

pathways, including MAP kinase, triggered by receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) [14, 21, 22]. Aside from ligand-dependent activation, EGFR activation may be caused by oxidative stress that is induced by activated neutrophils or exogenous H_2O_2, and this activation of EGFR may result in stimulation of mucin synthesis in NCI-H292 cells [3, 14].

In the present study, we first, examined which secreted mucin genes were induced by exogenous H_2O_2 in NHNE cells, including *MUC5AC, MUC5B, MUC6, MUC7* and *MUC19*. Second, we examined the signal pathway responsible for up-regulating these genes and confirmed that EGFR and ERK1/2 MAP kinase were associated with H_2O_2 stimulation in NHNE cells. Finally, we investigated and measured intracellular H_2O_2 generation after the stimulation of exogenous oxidative stress and examined which Nox subtype was involved in the endogenous generation of H_2O_2 in NHNE cells.

We found that exogenous H_2O_2 specifically induced only *MUC5AC* gene expression in a dose and time-dependent manners through the ligand-independent activation of EGFR and phosphorylation of ERK1 MAP kinase. Exogenous H_2O_2 did not induce *MUC5B, MUC6, MUC7* or *MUC19* expression. Additionally, exogenous H_2O_2 induced intracellular H_2O_2 generation through Nox4, one homolog of gp^{91phox} and inhibition of this intracellular H_2O_2 generation suppressed *MUC5AC* gene overexpression. This study provides the new insights that exogenous H_2O_2

generates intracellular H_2O_2 and that Nox4 may play critical roles at the downstream of EGFR and ERK1 MAP kinase in ROS-induced *MUC5AC* gene overexpression in chronic airway diseases.

II. MATERIALS AND METHODS

1. Materials

Hydrogen peroxide (H_2O_2) was purchased from Sigma Aldrich (St. Louis, MO). Anti-phospho-EGFR (Tyr1068), EGFR antibody, anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-total ERK MAP kinase, anti-phospho-p38 MAP kinase (Thr180/Tyr182), and anti-phospho-SAPK/JNK MAP kinase (Thr183/Tyr185) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-Nox4 antibody and ERK1 and ERK2 siRNA were purchased from Santa Cruz Biothechnology (Santa Cruz, CA, USA). Anti-α-tubulin antibody and GM6001 were purchased from Calbiochem (San Diego, CA, USA). Control siRNA (scramble RNA) for Nox4 was purchased from Dharmacon (Dharmacon Cat# D-001210-01-20) and siRNA for Nox4 was provided by Dr. Yun Soo Bae (Ewha Womans Univ, Seoul, Korea).

2. Cell culture

After the approval of the study protocol by the Institutional Review Board of the Yonsei University College of Medicine, we obtained the nasal mucosa samples from the middle turbinate of healthy patients. Normal human nasal epithelial (NHNE) cells were cultured as described previously [23, 24]. In brief, 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 size, transwell-clear (Costar Co, Cambridge, MA, USA) culture insert. Cells were cultured in a 1:1 mixture of basal epithelial growth medium (BEGM) and

Dulbecco's Modified Eagle's Medium (DMEM), containing all the supplements described previously [24]. 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 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 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 (FBS) and penicillin/streptomycin at 39°C in a humidified chamber with 5% CO2. Cells were grown to confluence in 6-well plates (Falcon, Flanklin Lakes, NJ). For deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI 1640 with 0.2% FBS to deprive them of serum [9].

3. Experimental conditions

H_2O_2 was diluted in PBS to stock concentrations of 100, 250, 500 and 1000 mM. The H_2O_2 stocks were further diluted in TBE to experimental concentrations of 100, 250, 500 and 1000 μM. NHNE cells were treated with H_2O_2 (100, 250, 500, and 1000 μM) for 2, 4, 8, 12, and 24 hrs for dose- and time-dependent studies, and the expression of *MUC5AC, MUC5B, MUC6, MUC7,* and *MUC19* were evaluated using reverse transcriptase PCR (RT-PCR). For western blot or intracellular H_2O_2

assays, cells were treated with 250 μM H_2O_2 for 5, 10, 30, 60, 120, and 180 min

4. RT-PCR

Total RNA was isolated from NHNE cells treated with H_2O_2 (100, 250, 500, and 1000 μM) 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, MUC5B, MUC6, MUC7,* and *MUC19* Genbank TM sequences and Nox subunit sequences (Table 1, 2). We used comparative kinetic analysis to compare mRNA levels of each gene for each set of culture conditions described previously [(24)]. PCR products were resolved on 2% agarose gels (FMC, Rockland, ME) and visualized with ethidium bromide under a transilluminator. When reverse transcriptase was omitted, no PCR products were observed, confirming that the amplified products were from mRNA and not genomic DNA contamination. The specific amplification of target genes was confirmed by sequencing the PCR products (dsDNA Cycle Sequencing System; GibcoBRL, Rockville, MD).

Table 1. Polymerase chain reaction (PCR) experimental conditions and oligonucleotide sequences.

Product	Cycle	Annealing Temp (^{o}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
MUC6	35	55	Forward	TCA CCT ATC ACC ACA CAA C
			Reverse	GGA GAA GAA GGA AAA AGA G
MUC7	35	55	Forward	CCA CAC CTA ATT CTT CCC
			Reverse	CTA TTG CTC CAC CAT GTC
MUC19	30	55	Forward	TTT AGA GGC ACT GGG ACC AC
			Reverse	ACC ATT GCC CAA AGA AGT TG
*β2-M	23	55	Forward	CTCGCCCTACTCTCTCTTTCTGG
			Reverse	GCTTACATGTCTCGATCCCACTTAA

β2-M : β2 microglobulin

Table 2. Polymerase chain reaction (PCR) experimental conditions and oligonucleotide sequences.

Product	Size (bp)	Cycle	Annealing Temp (^{o}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

5. Real time-PCR

Primers and probes were designed with PerkinElmer Life Sciences Prime Express® software and purchased from PE Biosystems. Commercial reagents (Taqman PCR Universal PCR Master Mix, PerkinElmer Life Sciences) were used according to the manufacturer's protocol. 1 μg of cDNA (reverse transcription mixture), oligonucleotide primers at a final concentration of 800 nM and 200 nM TaqMan hybridization probes were incubated in a 25μl volume. The real time-PCR

probe was labeled with carboxyfluoroscein (FAM) at the 5' end and the quencher carboxytetramethylrhodamine (TAMRA) at the 3' end. The MUC5AC and β2-microglobulin primers and TaqMan probe used are described in Table 3. Real time-PCR was performed using a PerkinElmer Life Sciences ABI PRISM® 7700 Sequence Detection System. The parameters were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The relative *MUC5AC* mRNA quantity was obtained using a comparative cycle threshold method and was normalized to β2-microglobulin as an endogenous control.

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

Product	**Primer**	**Oligonucleotide Sequence**
MUC5AC	Forward	5'-CAGCCACGTCCCCTTCAATA-3'
	Reverse	5'-ACCGCATTTGGGCATCC-3'
	Taqman probe	6FAM-CCACCTCCGAGCCCGTCACTGAG-TAMRA
*β2-M	Forward	5'-CGCTCCGTGGCCTTAGC-3'
	Reverse	5'-GAGTACGCTGGATAGCCTCCA-3'
	Taqman probe	6FAM-TGCTCGCGCGCTACTCTCTCTTTCTGGC-TAMRA

β2-M : β2 microglobulin

6. Western blot analysis

NHNE cells were grown to confluence in 6-well plates. After treatment with 250 μM H_2O_2 for 5, 10, 30, or 60 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 lysate were resolved using 10% SDS-PAGE and transferred to a PVDF membrane in Tris-buffered saline (TBS; 50 mM Tris-Cl (pH 7.5), 150 mM NaCl) for 90 or 180 min 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).

7. Cell trnsfection with ERKI or ERK2 isRNA

Specific siRNA (Santa Cruz) against ERK1 and ERK2 was used to suppress their respective expressions. The transfection rates of ERK1 or ERK2 siRNA were verified to be over 90% in NCI-H292 cells. 1ug of each siRNA and 1λ Lipofectamine 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, cellular deprivation was performed according to usual methods [(9)]. The same procedure was performed with **c**ontrol siRNA (Santa Cruz, sc-37007)**.**

8. Intracellular H_2O_2 assay

Intracellular ROS production was assessed as reported by Ohba et al (25). The 100 πΩ dishes of confluent cells were stimulated with H_2O_2 (250 μM) for 30, 60, 120, and 180 min, washed with modified Eagle's medium (HBSS) without phenol red and incubated for 10 min in the dark in Krebs-Ringer solution containing 5 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative DCFH and trapped within the cells [(26)]. 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 H_2O_2. Culture dishes were viewed on a Zeiss Axiovert 135 inverted microscope (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 then measured by Karl Zeiss vision system (KS400, version 3.0). The relative fluorescence intensity was taken as the average of the seven values and then, the mean relative fluorescence intensities were compared with each groups. All experiments were repeated at least three times.

9. Immunocytochemistry and immunofluorescence studies

Cytospin slides for immunostaining, were made on the second day after

confluency using NHNE cells and immunostaining was performed using anti-Nox4 antibody. The samples were fixed and incubated with rabbit anti-Nox4 antibody (diluted 1:100 in the PBS) overnight at 4℃. The samples were then washed repeatedly with PBS and incubated with a secondary FITC-conjugated affinity-purified goat anti-rabbit IgG (1:250) for 1 hr at room temperature. After extensive washing, glass cover slides were mounted and examined with a confocal microscope. The same procedures were performed using non-immunized mouse IgG (purified IgG, Sigma) instead of primary antibody as a negative control.

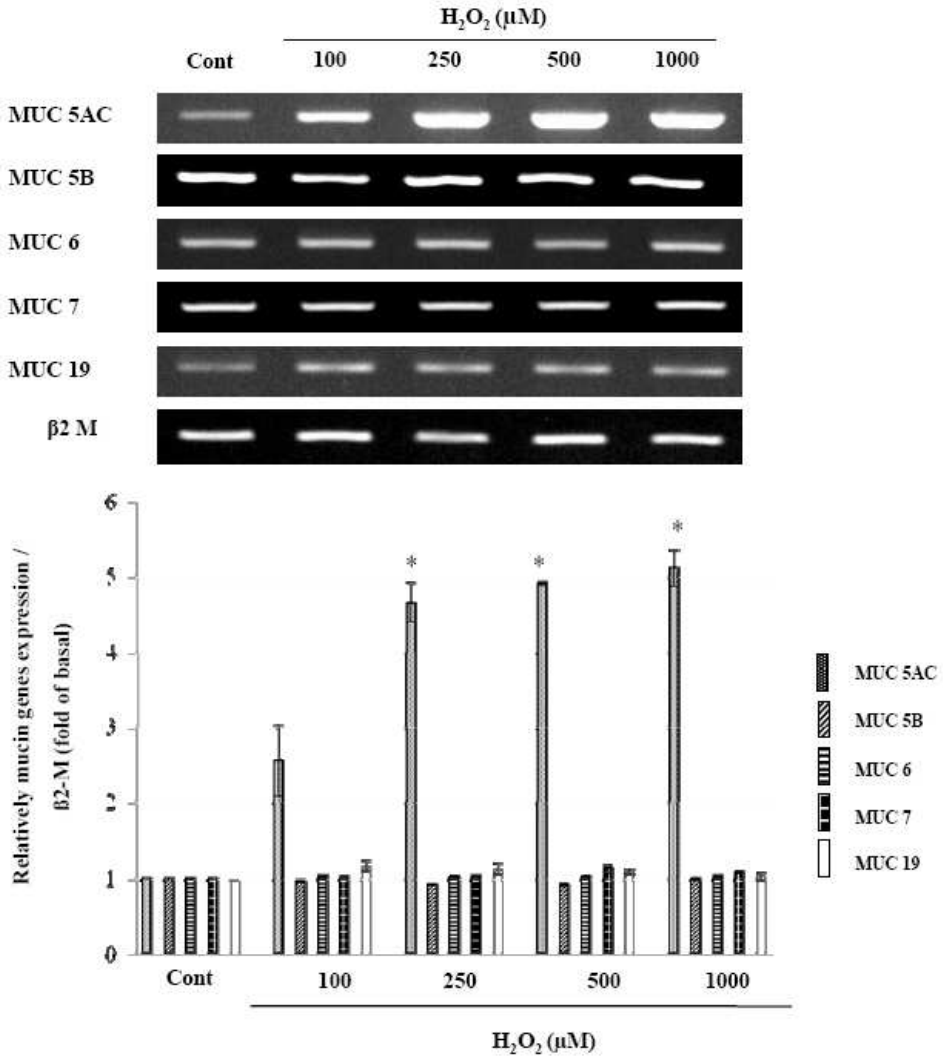
10. Statistical analysis

Data are expressed as means $\pm$ standard deviation (SD). At least three separate experiments were performed for each measurement with different cell lines or different NHNE cells. Differences between treatment groups were assessed by analysis of variance (ANOVA) with post hoc test. Differences were considered statistically significant at $p < 0.05$.

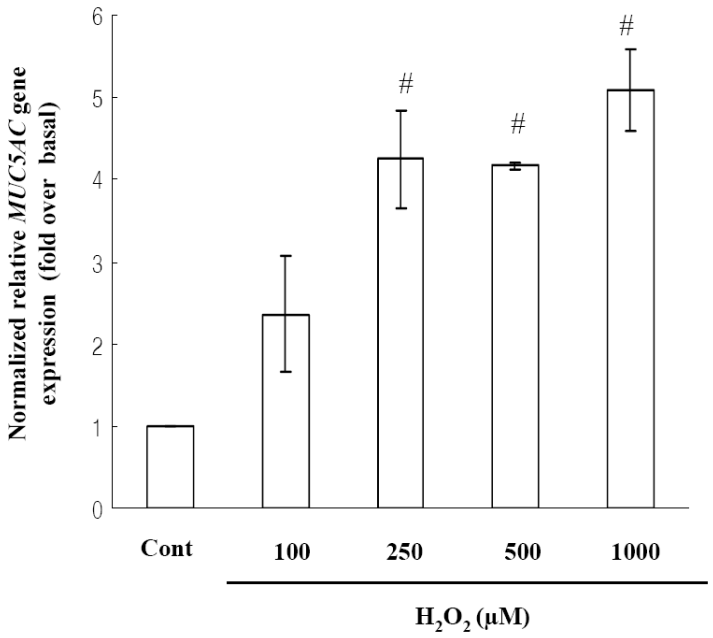
III. RESULTS

1. H_2O_2 induces *MUC5AC* gene expression in a dose and time-dependent manner, but not *MUC5B, MUC6, MUC7* or *MUC19* expression

To examine which secreted mucin genes could be induced by exogenous H_2O_2, RT-PCR was performed after treating cells (1 x 10^6/ml) with H_2O_2 (100, 250, 500, and 1000 μM) for 24 hrs. *MUC5AC* mRNA levels increased after H_2O_2 treatment in a dose-dependent manner, but *MUC5B, MUC6, MUC7, and MUC19* mRNA levels did not (Figure 1A). This showed that among secreted mucin genes, exogenous H_2O_2 specifically induces *MUC5AC* gene expression. Real-time PCR revealed that *MUC5AC* gene expression was significantly higher after treatment with 250 μM (4.25$\pm$0.59 fold over control; $p<0.05$), 500 μM (4.17$\pm$0.04 fold over control; $p<0.05$), and 1 mM H_2O_2 (5.08$\pm$0.49 fold over control; $p<0.05$) (Figure 1B). After H_2O_2 treatment for 2, 4, 8, 12, and 24 hrs, we carried out real-time PCR. *MUC5AC* gene expression was higher starting from 8 hr after treatment (8 hr: 4.47$\pm$0.10, 12 hr: 4.94$\pm$0.15, 24 hr: 4.86$\pm$0.02 fold over control; $p<0.05$; Figure 1C). No corresponding change was found in β2-microglobulin expression (the internal control). We used 250 μM of H_2O_2 for all subsequent experiments


H2O2 (μM)
Cont
100
250
500
1000
MUC 5AC
MUC 5B
MUC 6
MUC 7
MUC 19
β2 M
Relatively mucin genes expression / β2-M (fold of basal)
0
1
3
4
5
6
*
*
*
MUC 5AC
MUC 5B
MUC 6
MUC 7
MUC 19
Cont
100
250
500
1000
H2O2 (μM)

(A)


Normalized relative MUC5AC gene expression (fold over basal)
0
1
2
3
4
5
6
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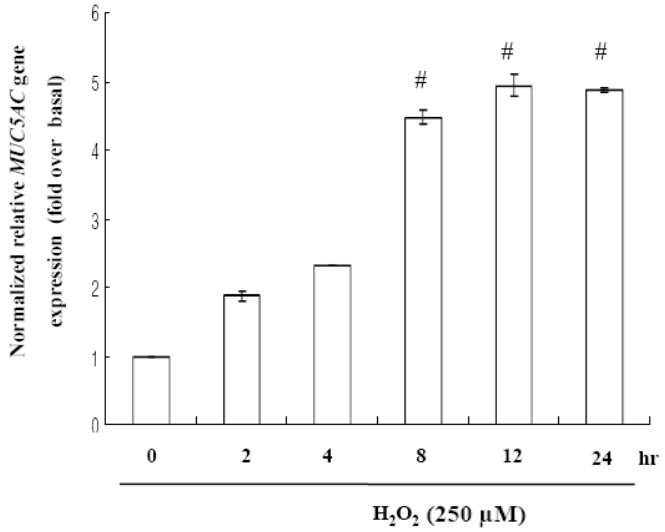
#
Cont
100
250
500
1000
H2O2 (μM)

(B)


Normalized relative MUC5AC gene expression (fold over basal)
0
1
2
3
4
5
6
#
#
#
0
2
4
8
12
24
hr
H2O2 (250 μM)

(C)

Figure 1. H_2O_2-induced mucin gene expressions. (A) Confluent NHNE cells were treated with H_2O_2 (100 μM, 250 μM, 500 μM, or 1 mM) for 24 h. Cont, control. β2-microglobulin (β2 M) was used as an internal control. (*: $p < 0.05$ when compared with control) (B) Real-time PCR demonstrating the dose-dependent effect of H_2O_2 on *MUC5AC* gene expression after 24 hr. (C) Real-time PCR demonstrating the time-dependent effect of H_2O_2 (250 μM) on *MUC5AC* gene expression. The results are from three separate experiments (Values are mean + SD, # : $p < 0.05$ when compared with control).

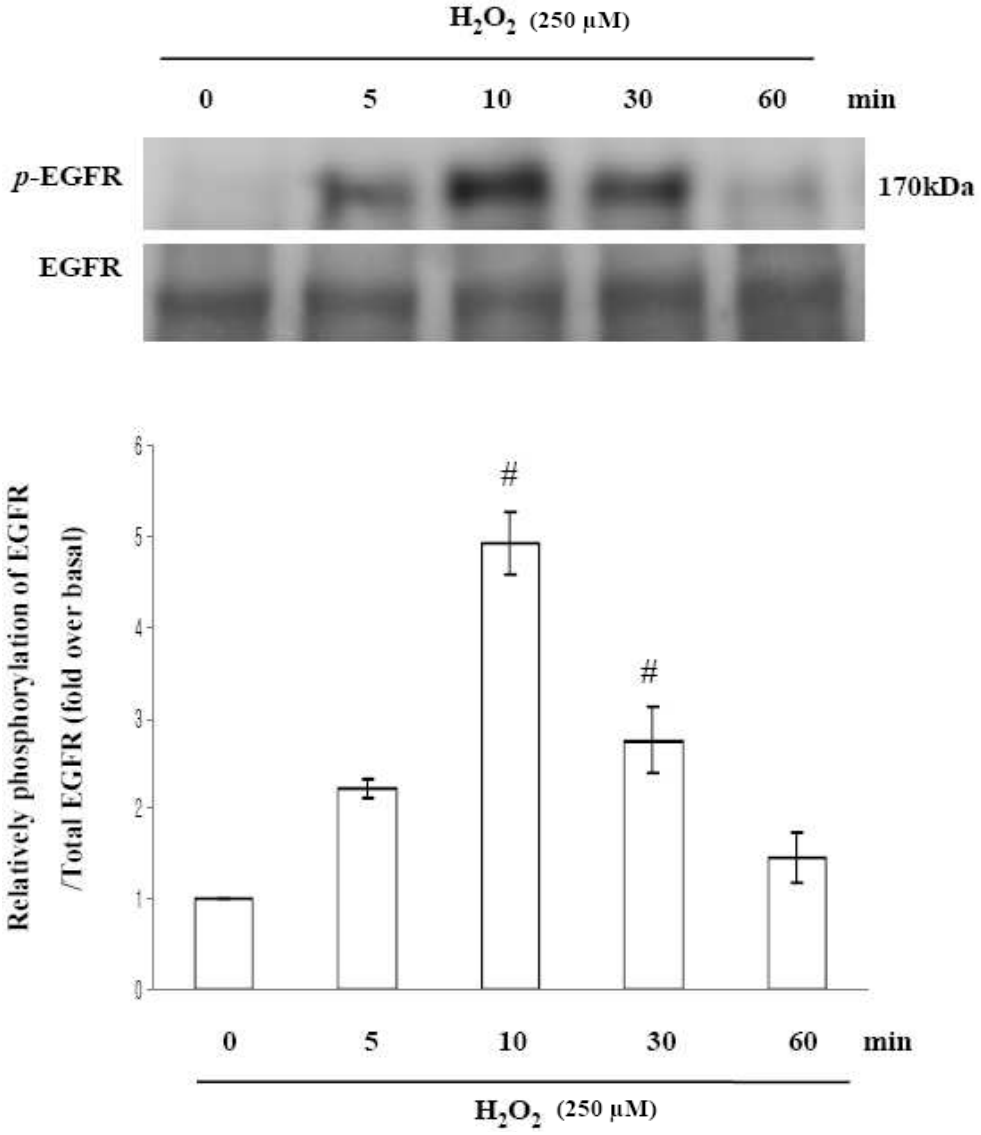
2. Exogenous H_2O_2 induces *MUC5AC* gene expression through the ligand-independent activation of EGFR

It is well known that EGFR is activated by oxidative stress and influence *MUC5AC* overexpression in NCI-H292 cells [14]. We examined whether EGFR was activated after stimulation by exogenous H_2O_2 in NHNE cells. Maximum activation of EGFR occurred 10 min (4.91±0.07 fold over control) after stimulation of exogenous H_2O_2 (250 μM) and gradually decreased thereafter (Figure 2A). We next evaluated the mechanism behind EGFR activation. NHNE cells were treated with galardin (GM6001), a broad-spectrum matrix metalloproteinase inhibitor, in a dose-dependent manner (1, 10, 30 μM) for 1 hr and then stimulated with exogenous H_2O_2. Pretreatment of NHNE cells with galardin did not inhibit increased phosphorylation of EGFR after stimulation with exogenous H_2O_2 (4.73±0.34 versus 4.48±0.29, 4.53±0.35, 4.37±0.16 fold over control, respectively, Figure 2B), indicating that exogenous H_2O_2 increased the phosphorylation of EGFR through a ligand-independent mechanism.

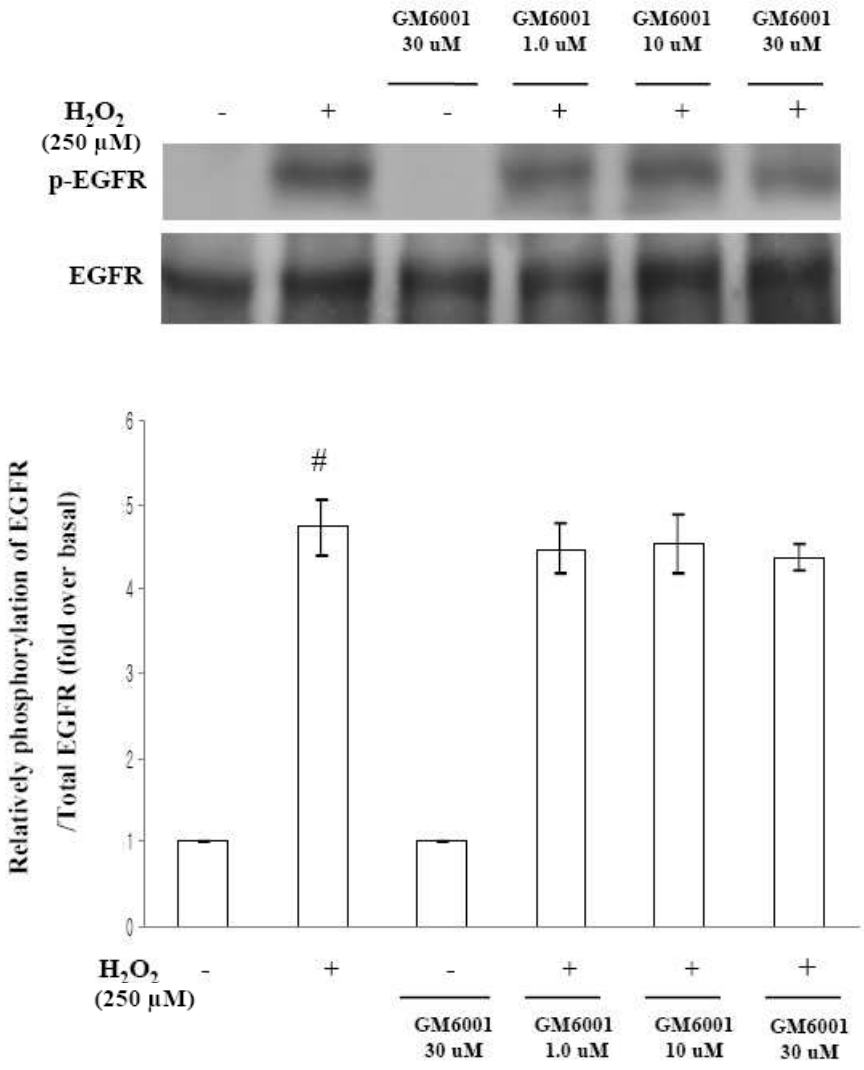
3. Phosphorylation of EGFR and ERK1 MAP kinase is involved in exogenous H_2O_2 -induced *MUC5AC* gene expression

As a next step, we examined the involvement of the MAP kinase signal pathway, as a downstream signal of EGFR. ERK1/2 MAP kinase was maximally activated after 10 min of stimulation with exogenous H_2O_2 (4.71±0.71, fold over control; $p<0.05$) and gradually decreased thereafter in NHNE cells (Figure 2C). In order to investigate the involvement of EGFR and ERK1/2 MAP kinase in H_2O_2-induced *MUC5AC* gene expression, we first pretreated NHNE cells with AG1478 (10 μM), tyrosine kinase inhibitor, for 1 hr before treating with H_2O_2. Western blot analysis and real time-PCR clearly showed that AG1478 pretreatment inhibited the phosphorylation of ERK1/2 MAP kinase and H_2O_2-induced *MUC5AC* gene expression (4.80±0.58 versus 1.61±0.16, fold over control; $p<0.05$; Figure 2D).

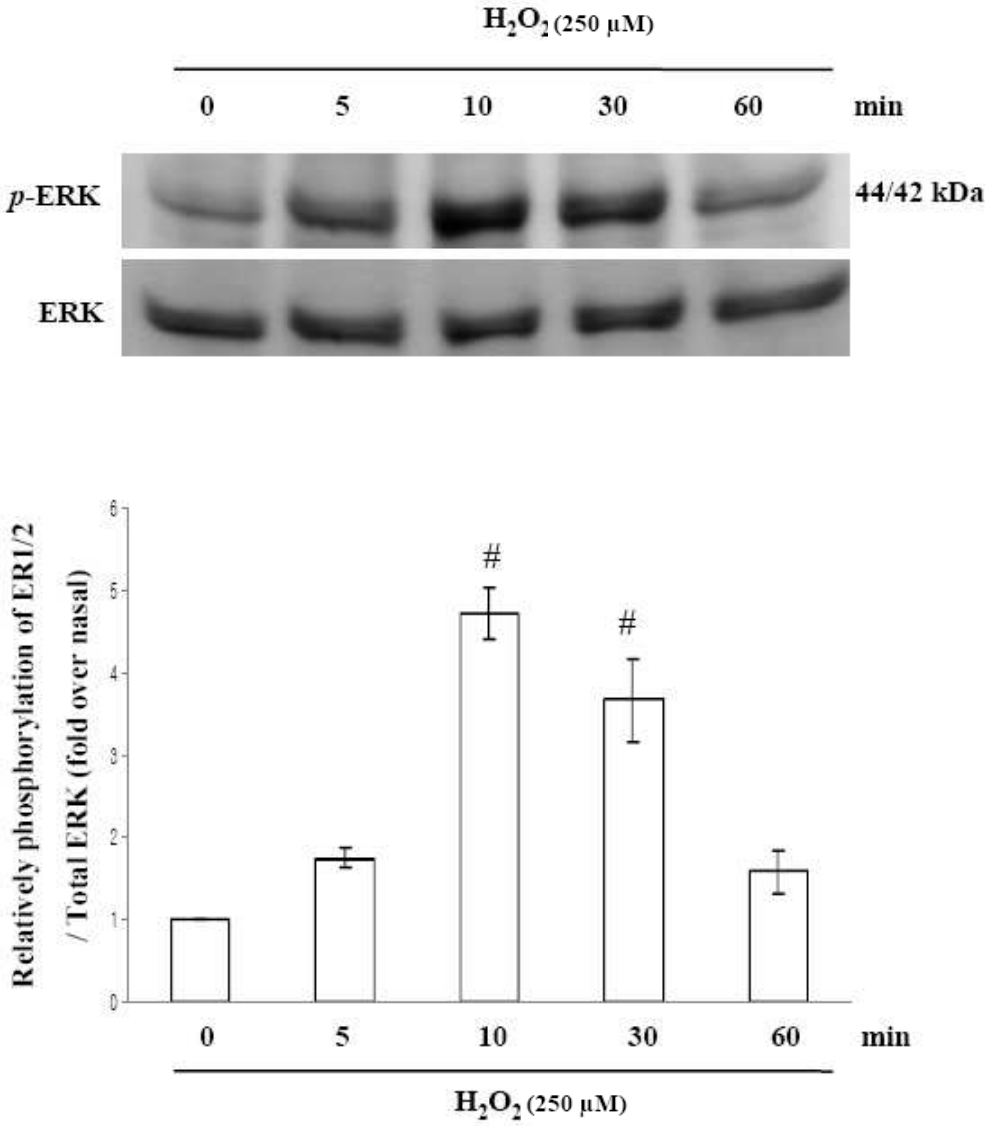
To study the specificity of ERK1/2 MAP kinase, cells were transfected transiently with ERK1 or ERK2 MAP kinase siRNA, respectively. Interestingly, transfection with ERK1 siRNA specifically reduced the exogenous H_2O_2-induced *MUC5AC* gene expression compared with transfection with **c**ontrol siRNA (sc-37007) (4.67±0.70 versus 1.82±0.10 fold over control; $p<0.05$), while, transfection with ERK2 siRNA did not (4.67±0.70 versus 4.11±0.18 fold over control, Figure 2E). These results indicate that exogenous H_2O_2-induced *MUC5AC* gene expression requires the activation of EGFR and subsequent phosphorylation of ERK1 MAP kinase in NHNE cells.


H2O2 (250 μM)
0
5
10
30
60
min
p-EGFR
170kDa
EGFR
Relatively phosphorylation of EGFR
/Total EGFR (fold over basal)
#
#
0
1
2
3
4
5
6
0
5
10
30
60
min
H2O2 (250 μM)

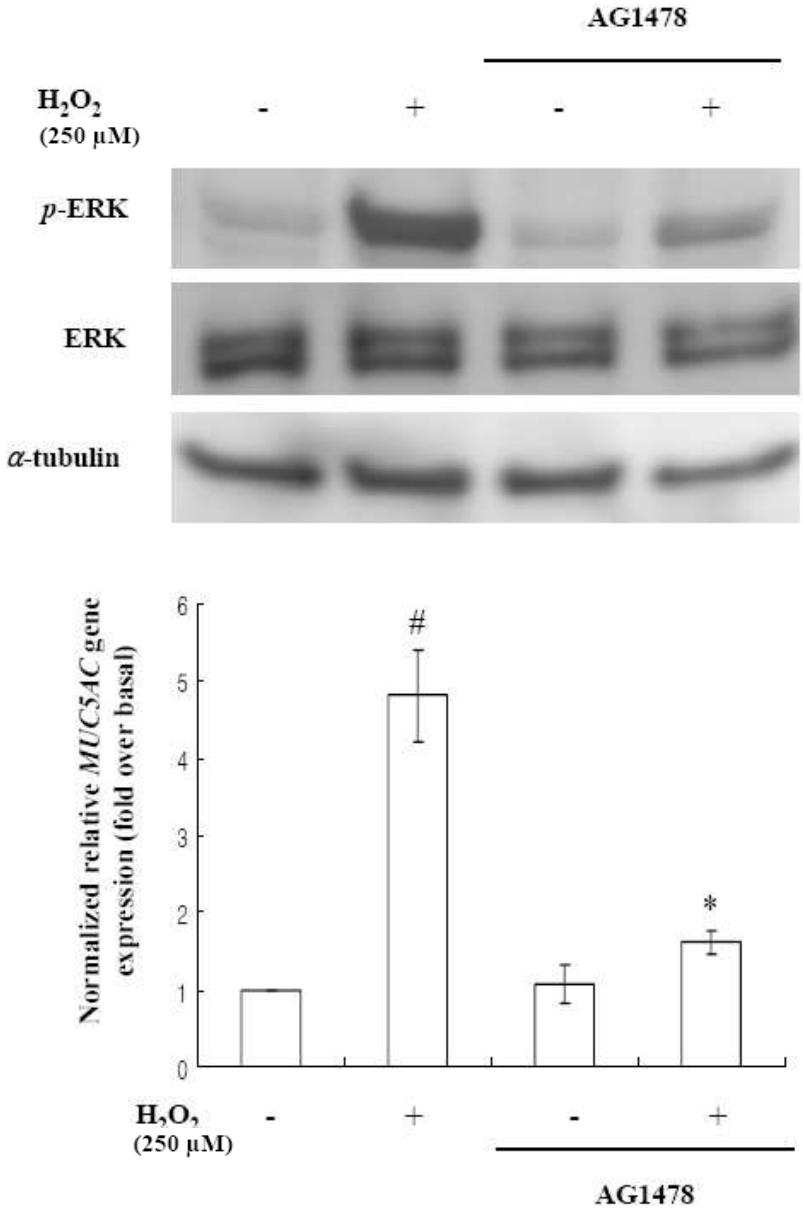
(A)


GM6001 30 uM
GM6001 1.0 uM
GM6001 10 uM
GM6001 30 uM
H2O2 (250 μM)
- + - + + +
p-EGFR
EGFR
Relatively phosphorylation of EGFR /Total EGFR (fold over basal)
#
0 1 2 3 4 5 6
H2O2 (250 μM)
- + - + + +
GM6001 30 uM
GM6001 1.0 uM
GM6001 10 uM
GM6001 30 uM

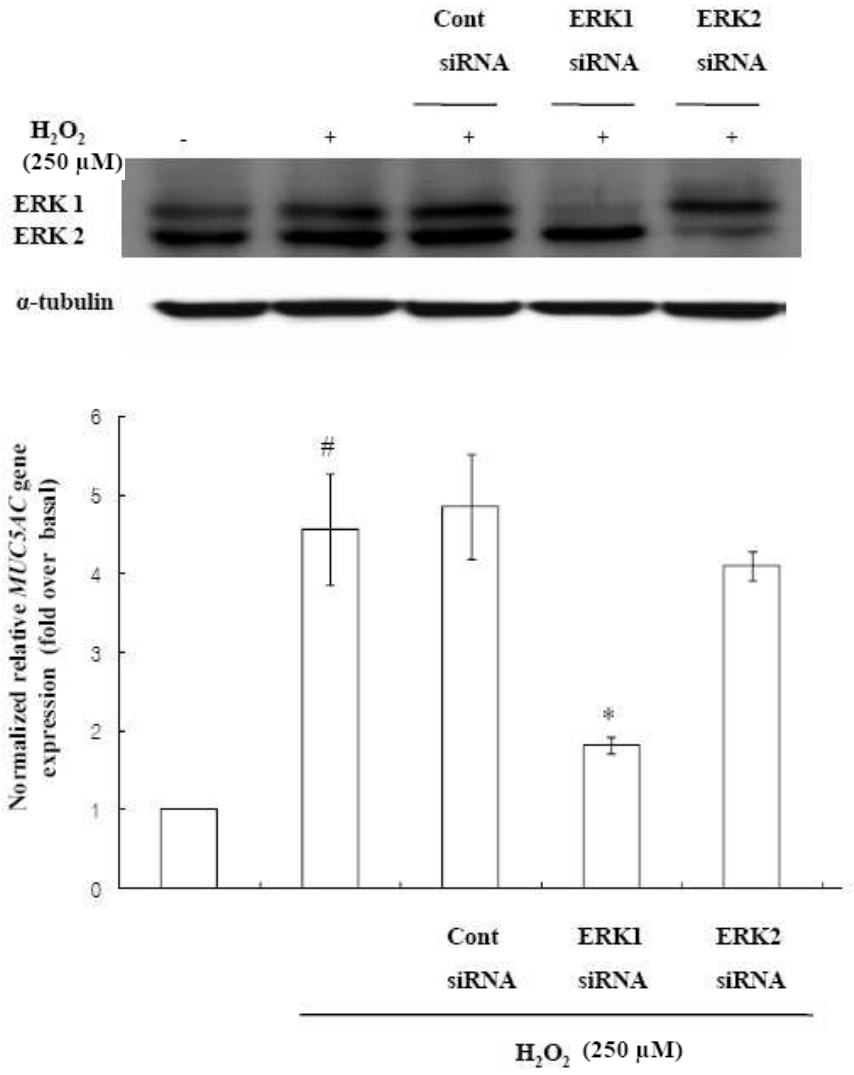
(B)


H2O2 (250 μM)
0
5
10
30
60
min
p-ERK
44/42 kDa
ERK
Relatively phosphorylation of ER1/2
/ Total ERK (fold over nasal)
#
#
0
1
2
3
4
5
6
0
5
10
30
60
min
H2O2 (250 μM)

(C)


AG1478
H2O2
(250 μM)
-
+
-
+
p-ERK
ERK
α-tubulin
Normalized relative MUC5AC gene expression (fold over basal)
0
1
2
3
4
5
6
#
*
H2O2
(250 μM)
-
+
-
+
AG1478

(D)



(E)

Figure 2. Effect of exogenous H_2O_2 on the activation EGFR and ERK1 MAP kinase. (A) NHNE cells were treated with H_2O_2 (250 μM) for 5, 10, 30, or 60 min. Control cells were maintained in basal growth medium with PBS. Western blot

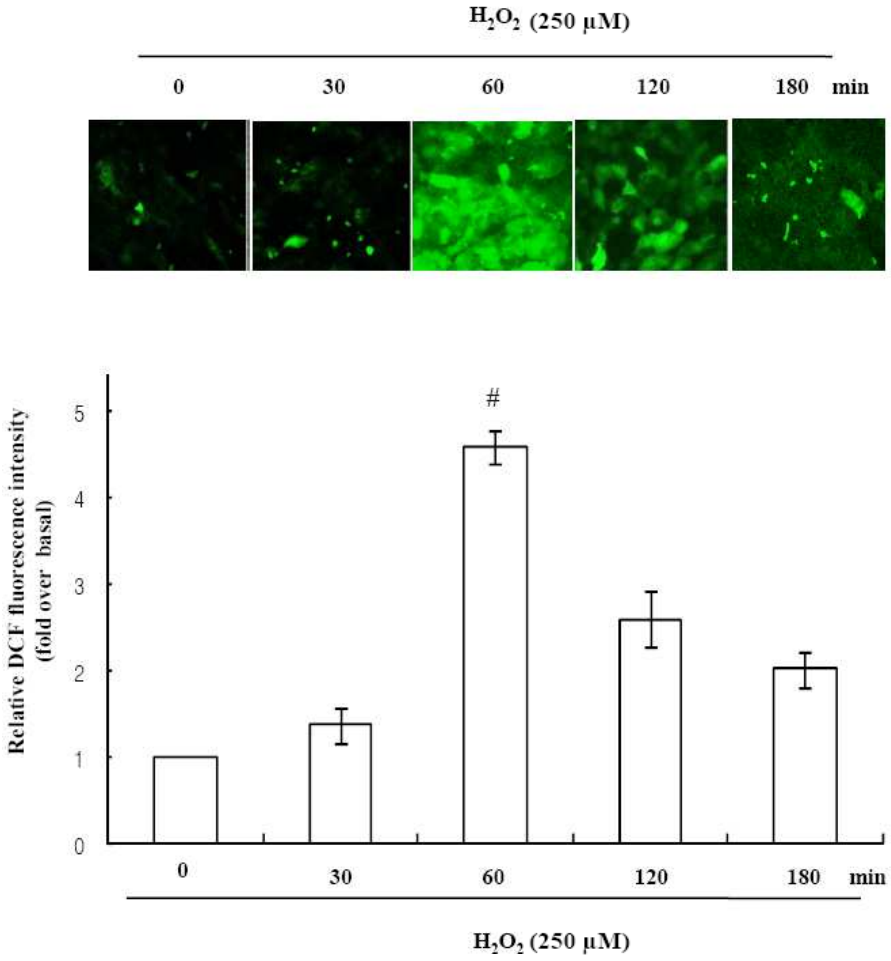
analysis demonstrated the effect of H_2O_2 on EGFR. (B) NHNE cells were treated with galardin (GM6001, 1, 10, 30 μM) for 1 hr before treatment of H_2O_2 (250 μM). Western blot analysis showed that pretreatment with galardin did not inhibit the increased phosphorylation of EGFR. (C) NHNE cells were treated with H_2O_2 (250 μM) for 5, 10, 30, or 60 min. Western blot analysis showed that exogenous H_2O_2 increased the phosphorylation of ERK1/2 MAP kinase. (D) NHNE Cells were treated with AG1478 (10 μM) for 30 min, then stimulated with H_2O_2 (250 μM) for 10 min. Western blot analysis and real-time PCR showed the effect of AG1478. (E) Western blot and real-time PCR showed the expressions of ERK1, ERK2 and *MUC5AC* gene expression after the transfection with ERK1 and ERK2 siRNA respectively. The results of Western blot analyses are representative of three separate experiments and the results of densitometry and real-time PCR are from three separate experiments (Values are mean $\pm$ SD, $^{\#}p < 0.05$ when compared with control, $^{*}p < 0.05$ when compared with H_2O_2 treatment group).

4. Exogenous H_2O_2 induces intracellular H_2O_2 generation through NADPH oxidase

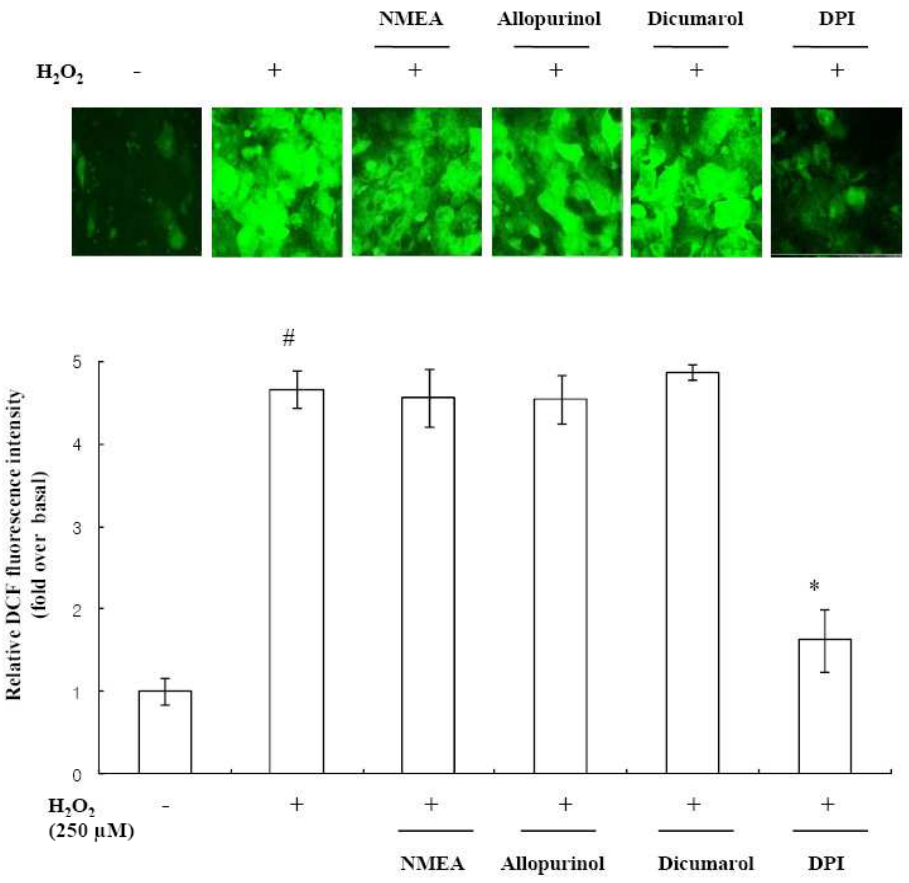
NHNE cells were stimulated with media containing 250 μM H_2O_2 for 30, 60, 120, and 180 min. The production of H_2O_2 was measured using a fluorescence-based assay with 2',7'- DCFH-DA and laser-scanning confocal microscopy. The stimulation of NHNE cells with H_2O_2 resulted in a time-dependent increase in the intensity of DCF fluorescence, with the maximal increase (4.6-fold) apparent 60 min after stimulation. Fluorescence had diminished by 180 min (Figure 3A).

We next examined whether Nox is involved in exogenous H_2O_2–induced intracellular H_2O_2 generation. To examine the involvement of other enzymes, we investigated the effect of each inhibitors of Nox (Diphenyleneiodium chloride, DPI Sigma), NO synthase (N^G-Monoethyl-L-arginine, NMEA, Calbiochem), xanthine oxidase (allopurinol, Sigma), and NADPH:quinine oxidoreductase (dicumarol, Sigma). After pretreating NHNE cells with DPI 30 uM, NMEA 10 uM, allopurinol 100 uM or dicumarol 30 uM, we measured the change of intracellular H_2O_2 and performed real-time PCR to examine *MUC5AC* gene expression. NMEA, allopurinol and dicumarol did not have a significant inhibitory effect on exogenous H_2O_2-induced intracellular H_2O_2 or *MUC5AC* gene overexpression (Figure 3B, 3C). In contrast, pretreatment with DPI, suppressed exogenous H_2O_2-induced intracellular H_2O_2 (4.66 ± 0.22 versus 1.62 ± 0.38 fold over control, $p<0.05$) and

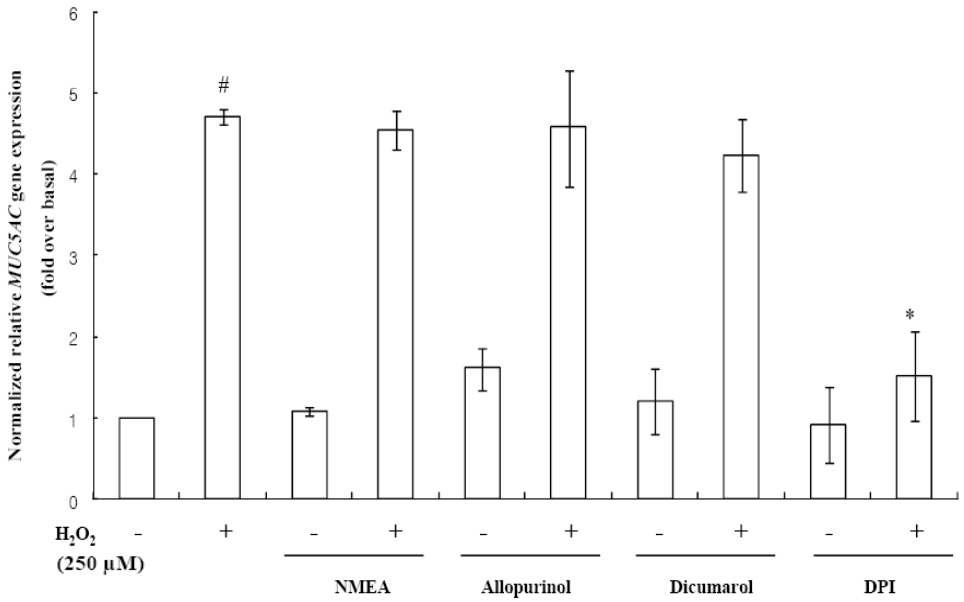
MUC5AC gene expression (4.72±0.89 versus 1.52±0.23 fold over control, $p<0.05$, Figure 3B, 3C). These results suggest that exogenous H_2O_2 can produce intracellular H_2O_2 through Nox and Nox may affect *MUC5AC* gene overexpression through the generation of intracellular H_2O_2.



(A)


NMEA
Allopurinol
Dicumarol
DPI
H2O2 - + + + + +
#
*
Relative DCF fluorescence intensity
(fold over basal)
0
1
2
3
4
5
H2O2
(250 μM)
- + + + + +
NMEA
Allopurinol
Dicumarol
DPI

(B)



(C)

Figure 3. Production of intracellular H_2O_2 through Nox. (A) NHNE cells were stimulated with media containing 250 μM of H_2O_2 for 30, 60, 120, or 180 min and the production of intracellular H_2O_2 was measured using a fluorescence-based assay with 2', 7'-DCFH-DA and laser-scanning confocal microscopy. The figures of fluorescent intensity are representative of three separate experiments and the graph of intensity is from three separate experiments (Values are means $\pm$ SD, *: $p < 0.05$). (B) After pretreatment with NMEA (10 μM), allopurinol (100 μM), dicumarol (30

μM) and DPI (30 μM), we measured the change of intracellular H_2O_2. (C) Real-time PCR showed that the effect of each inhibitors on the exogenous H_2O_2 (250 μM)-induced *MUC5AC* gene expression. Results are from three separate experiments with the different NHNE cells (Values are mean $\pm$ SD, $^{\#}p < 0.05$ when compared with control, $^{*}p < 0.05$ when compared with treatment group with H_2O_2).

5. Ligand-independent activation of EGFR and phosphorylation of ERK MAP kinase mediate intracellular H_2O_2 generation after stimulation with exogenous H_2O_2.

Next, we investigated whether EGFR and ERK1 MAP kinase are involved in exogenous H_2O_2-induced intracellular H_2O_2 generation. NHNE cells were pretreated with AG1478 (10μM), or an MEK1 inhibitor (PD98059; 30μM) for 30 min before stimulation with exogenous H_2O_2 for 60 min. Intracellular H_2O_2 production was measured using confocal fluorescence microscopy. The generation of intracellular H_2O_2 was suppressed after pretreatment with AG1478 (4.85$\pm$1.16 versus 1.31$\pm$0.28 fold over control, $p<0.05$) and PD98059 (4.85$\pm$1.16 versus 1.32$\pm$0.20 fold over control, $p<0.05$; Figure 4), demonstrating that exogenous H_2O_2-induced intracellular H_2O_2 generation was increased through the activation of EGFR and ERK1 MAP kinase, which may be involved in Nox-related generation of intracellular H_2O_2.

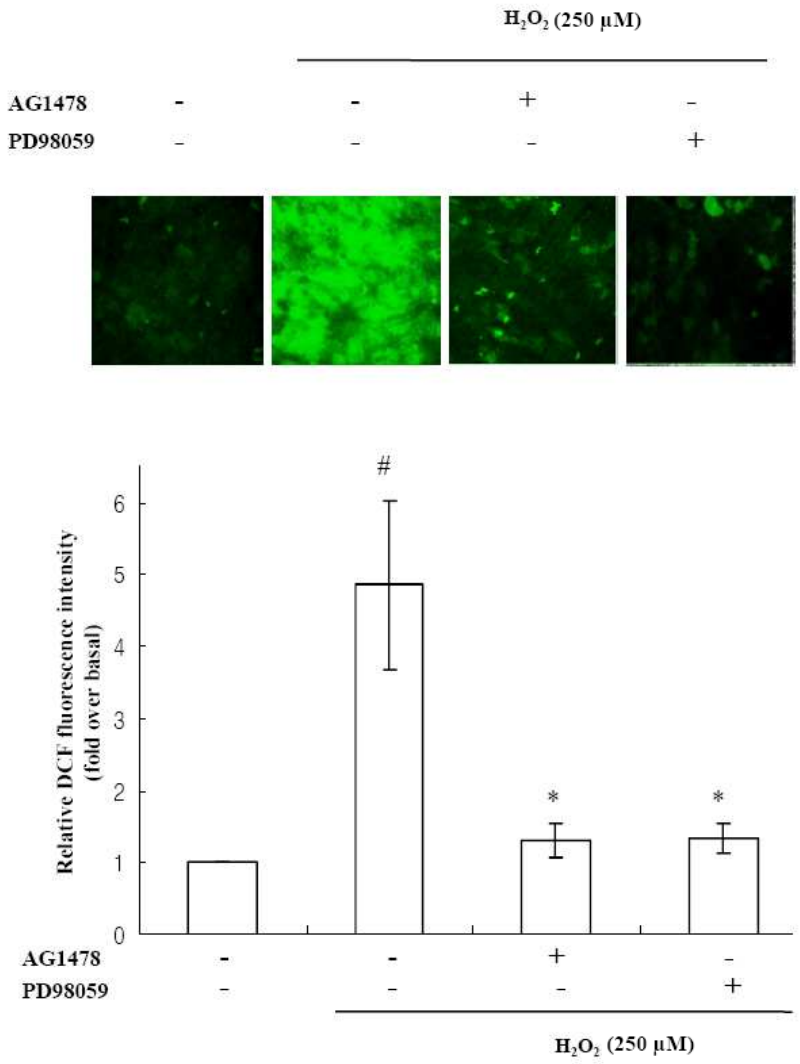


Figure 4. The role of EGFR and ERK1 MAP kinase in exogenous H_2O_2-induced intracellular H_2O_2 generation. NHNE cells were pretreated with AG1478 (10 μM) or PD98059 (30 μM) for 30 min, then stimulated with H_2O_2 (250μM) for 60 min. Intracellular H_2O_2 production was measured using a fluorescence-based assay with 2', 7'- DCFH-DA and laser-scanning confocal microscopy. Individual data points

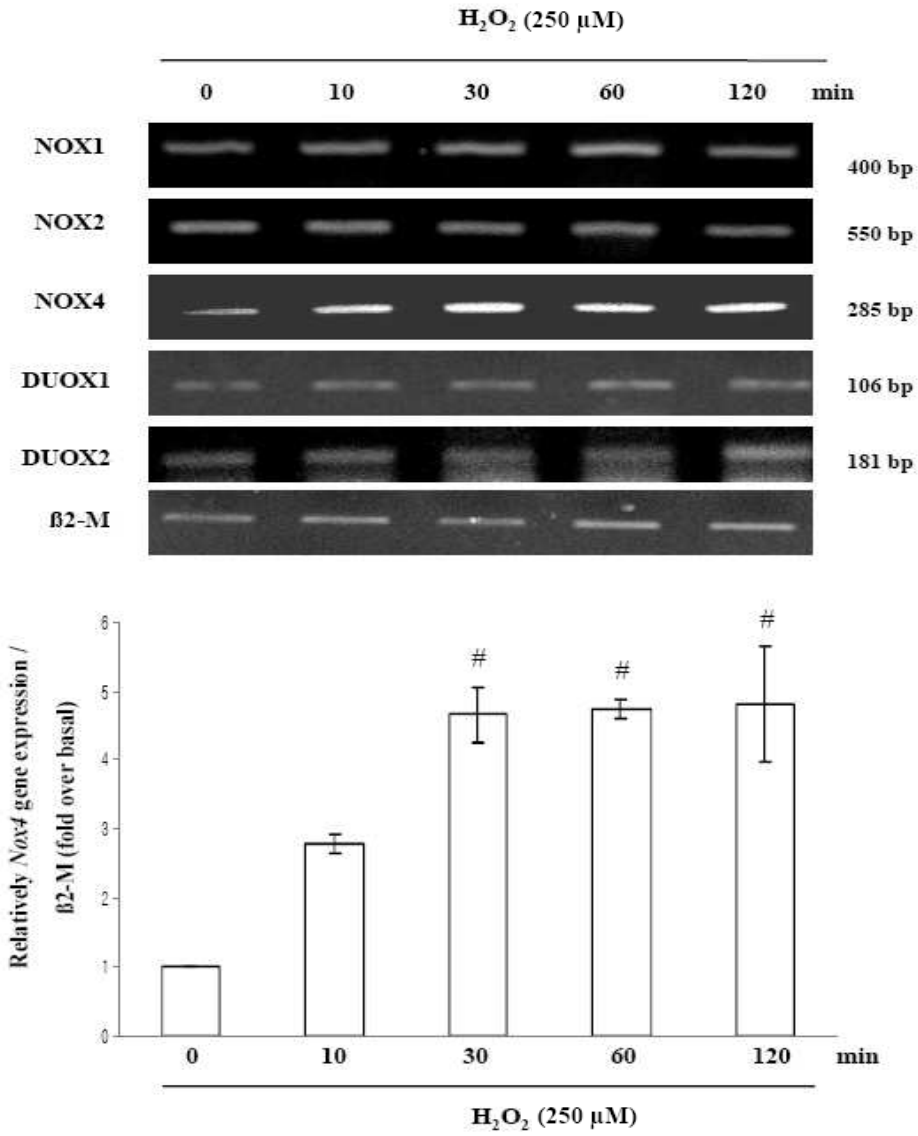
were quantified as fluorescent intensity units and are presented as a percentage of control. The figures of fluorescent intensity are representative of three separate experiments and the graph of intensity is from three separate experiments (Values are mean $\pm$ SD, $^{\#}p < 0.05$ when compared with control, $*p < 0.05$ when compared with treatment group with H_2O_2).

6. Nox4 is the primary Nox homolog involved in exogenous H_2O_2-induced intracellular H_2O_2 generation and *MUC5AC* gene overexpression in NHNE cells

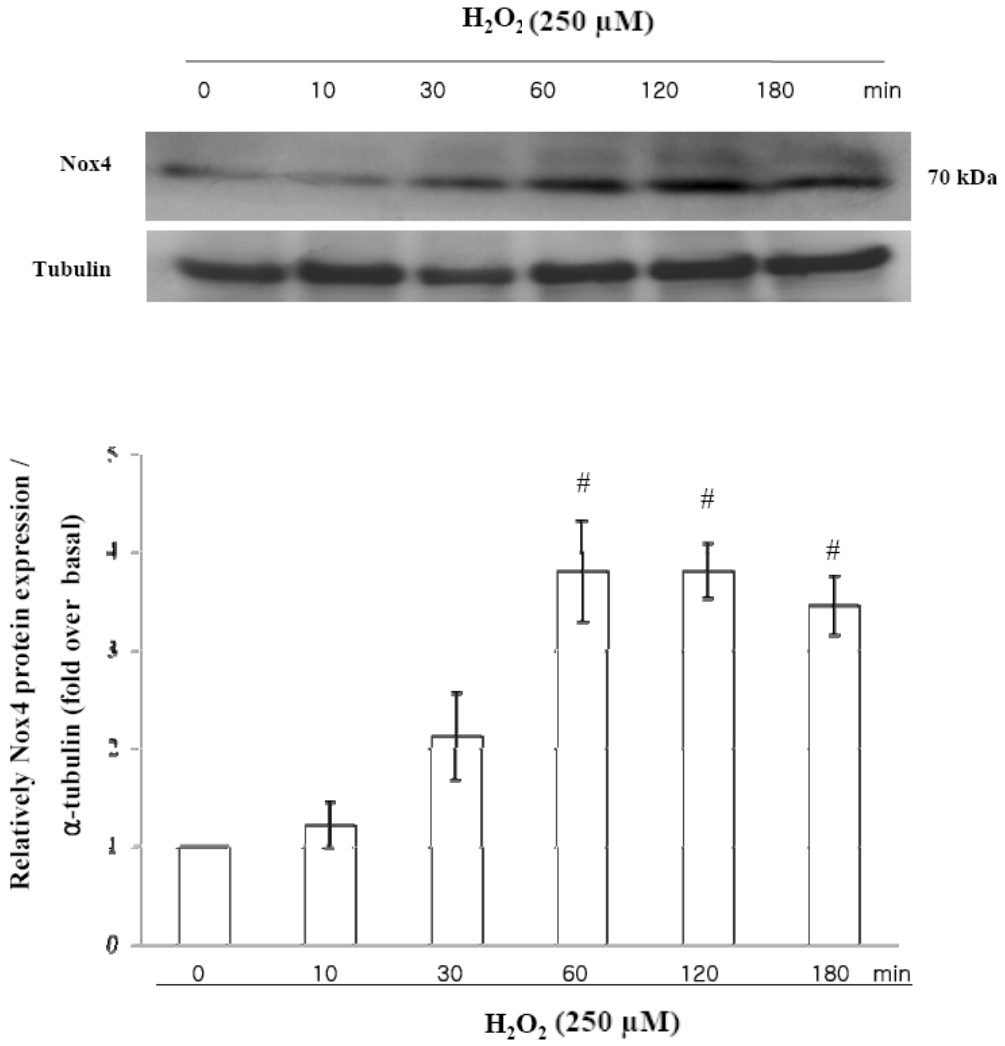
To determine whether H_2O_2 causes a significant increase in the expression of Nox subtypes and to identify which Nox subtypes may be involved in intracellular H_2O_2 generation within NHNE cells, cells were treated with exogenous H_2O_2 in a time-dependent manner and RT-PCR for Nox subtypes was performed. Interestingly, only *Nox4* gene expression increased significantly 30 min after stimulation with exogenous H_2O_2 (4.39$\pm$0.39 fold over control; $p<0.05$ Figure 5A). After stimulation with exogenous H_2O_2, gene expression of *Nox1, Nox2, Duox1* and *Duox2* did not increase, besides, Nox3 and Nox5 were not expressed in NHNE cells (Figure 5A). We next performed Western blot analysis to examine whether Nox4 protein is activated after stimulation by exogenous H_2O_2 in NHNE cells. The expression of Nox4 protein peaked at 60 min (3.56$\pm$0.44 fold over control $p<0.05$ Figure 5B) after stimulation with exogenous H_2O_2 (250 μM).

We examined transient transfection with Nox4 siRNA on NCI-H292 cells to verify the critical function of Nox4 in exogenous H_2O_2-induced intracellular H_2O_2 generation. Transfection with Nox4 siRNA specifically reduced exogenous H_2O_2-increased *Nox4* gene expression compared with transfection using control siRNA (Figure 6A). Importantly, our data showed that cells transfected with Nox4 siRNA,

did not undergo exogenous H_2O_2-induced intracellular H_2O_2 generation and inhibited *MUC5AC* gene overexpression, whereas cells transfected with control siRNA exhibited the expected increase in H_2O_2 and *MUC5AC* gene expression in response to exogenous H_2O_2 (Figure 6B, 6C). These results suggest that Nox4 is a critical Nox homolog in NHNE cells in response to exogenous H_2O_2 and is essential for exogenous H_2O_2-induced intracellular H_2O_2 generation and *MUC5AC* gene expression.


H2O2 (250 μM)
0
10
30
60
120
min
NOX1
NOX2
NOX4
DUOX1
DUOX2
ß2-M
400 bp
550 bp
285 bp
106 bp
181 bp
Relatively Nox4 gene expression / ß2-M (fold over basal)
#
#
#
0
1
2
3
4
5
6
0
10
30
60
120
min
H2O2 (250 μM)

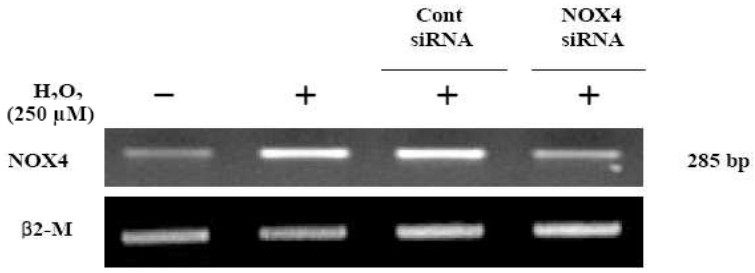
(A)



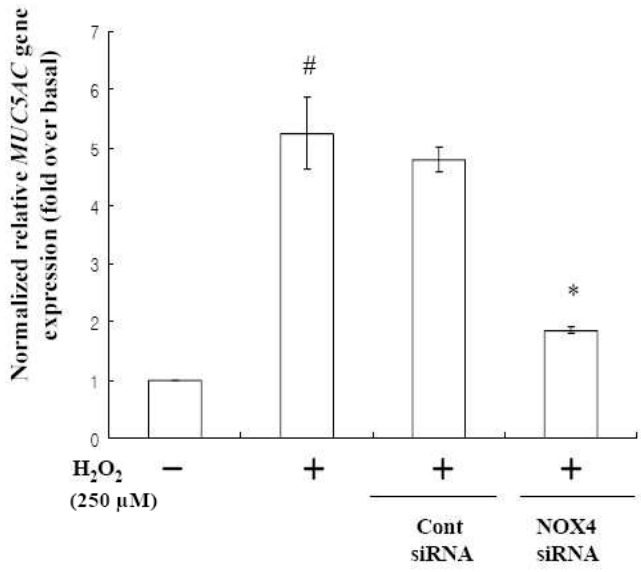
(B)

Figure 5. *Nox4* gene and protein expressions after stimulation with exogenous H_2O_2. NHNE cells were stimulated with exogenous H_2O_2 (250 μM) for 10, 30, 60, 120, 180 min. (A) RT-PCR showed that only *Nox4* gene expression increased significantly 30 min after stimulation with exogenous H_2O_2. (B) The maximum

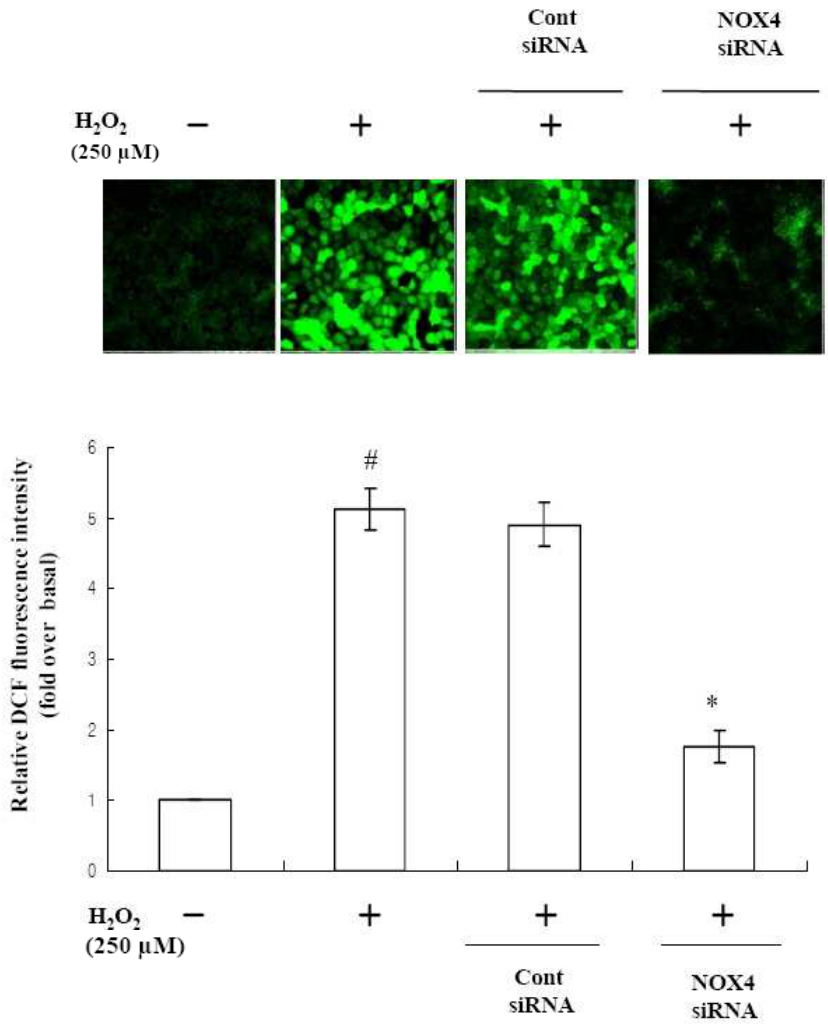
increase of Nox4 protein was observed at 60 min after stimulation with exogenous H_2O_2. The results of western blot analyses and RT-PCR are representative of three separate experiments and the results of densitometry are from three separate experiments (Values are mean ± SD, $^{\#}p < 0.05$ when compared with control)



(A)



(B)



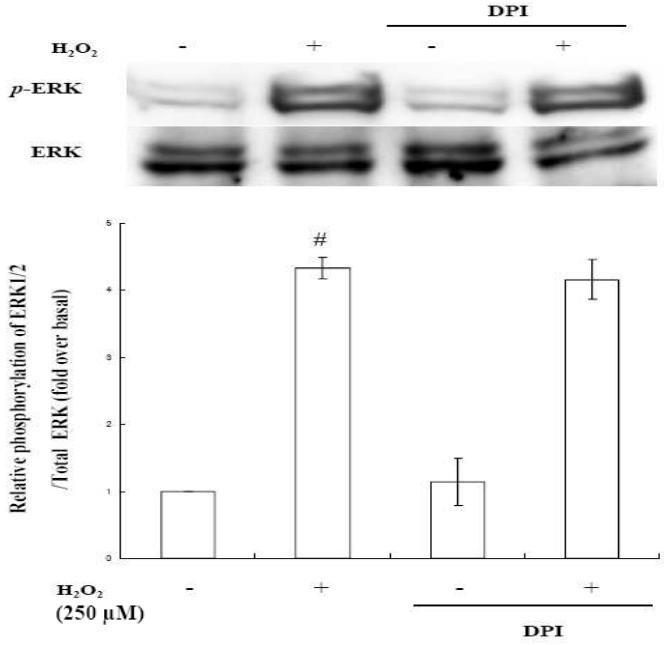
(C)

Figure 6. The role of Nox4 in exogenous H_2O_2-induced intracellular H_2O_2 generation and *MUC5AC* gene overexpression. (A) The increased Nox4 gene expression was suppressed after transfection of Nox4 siRNA. Similar results were obtained in three separate experiments. (B) Real time-PCR showed that *MUC5AC*

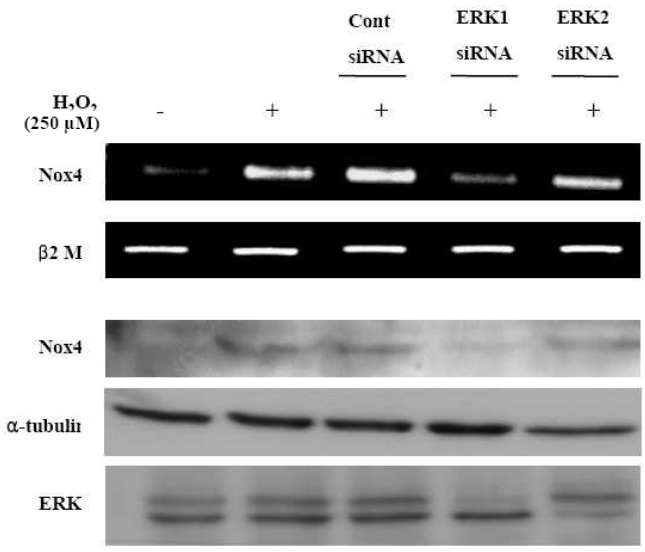
expression was suppressed after transfection with Nox4 siRNA. Results are from three separate experiments (Values are mean $\pm$ SD, $^{\#}p < 0.05$ when compared with control, * $p < 0.05$ when compared with treatment group with H_2O_2). (C) The increased fluorescence intensity after stimulation with exogenous H_2O_2 was reduced after transfection with Nox4 siRNA. The figures of fluorescent intensity are representative of three separate experiments and the graph of intensity is from three separate experiments (Values are means $\pm$ SD, $^{\#}p < 0.05$ when compared with control, $^{*}p < 0.05$ when compared with treatment group with H_2O_2)

7. Nox4 is involved in exogenous H_2O_2-induced intracellular H_2O_2 generation downstream of EGFR/ERK1.

Having established the role of Nox4 in exogenous H_2O_2-intracellular H_2O_2 generation and *MUC5AC* gene overexpression, we examined the signal transduction sequence among EGFR, ERK1 MAP kinase, and Nox4. NHNE cells were pretreated with DPI (30 μM) and stimulated with exogenous H_2O_2 (250 μM), followed by Western blot analysis. No significant change in the phosphorylation of ERK1/2 MAP kinase was found after DPI pretreatment compared to exogenous H_2O_2 stimulation alone (Figure 7A). Next, cells were transfected transiently with either ERK1 or ERK2 siRNA. Transfection with ERK1 siRNA specifically suppressed the exogenous H_2O_2-dependent induction of *Nox4* mRNA expression compared to control siRNA-transfection (4.66 ± 0.54 versus 1.33 ± 0.14 fold over control; $p<0.05$) and no change in *Nox4* gene expression was observed by transfection with ERK2 siRNA (4.66 ± 0.54 versus 4.61 ± 0.49 fold over control, Figure 7B). We obtained similar results in Western blot analysis. These results show that exogenous H_2O_2-induced *MUC5AC* gene expression requires the activation of EGFR, the phosphorylation of ERK1 MAP kinase and subsequent Nox4 overexpression in NHNE cells.


DPI
H_2O_2
-
+
-
+
p-ERK
ERK
Relative phosphorylation of ERK1/2
/Total ERK (fold over basal)
#
H_2O_2
(250 μM)
DPI

(A)


Cont
siRNA
ERK1
siRNA
ERK2
siRNA
H_2O_2
(250 μM)
Nox4
β2 M
Nox4
α-tubulin
ERK

(B)

Figure 7. Nox4 produced intracellular H_2O_2 through the activation of EGFR/ERK1. (A) After pretreatment with DPI (30μM), NHNE cells were stimulated with exogenous H_2O_2 (250 μM). Western blot analysis showed no significant change in the phosphorylation of ERK1/2 MAP kinase. (B) NCI-H292 cells were transfected with ERK1 and ERK2 siRNA. The results of Western blot analyses and RT-PCR are representative of three separate experiments. (Values are mean $\pm$ SD, $^{\#}p < 0.05$ when compared with control).

8. Nox4 may be localized in the cell membrane and the cytoplasm of NHNE cells.

It has been reported that Nox4 protein is localized in both paranuclear lesion and nuclear lesions of human aortic smooth muscle cells or human airway smooth muscle cells [27, 28]. The intracellular localization of Nox4 in NHNE cells was analyzed by immunofluorescence staining with anti-Nox4 antibody. Confocal microscopic findings revealed that Nox4 was expressed in the cell membrane and the cytoplasm (Figure 8, Right panels). No staining was detected when primary Nox4 antibody was omitted and replaced with purified IgG (Figure 8, Left panels). This finding suggests that after stimulation of exogenous H_2O_2, Nox4 protein in the cell membranes and cytoplasm may be activated, resulting in the production of intracellular H_2O_2 in NHNE cells.

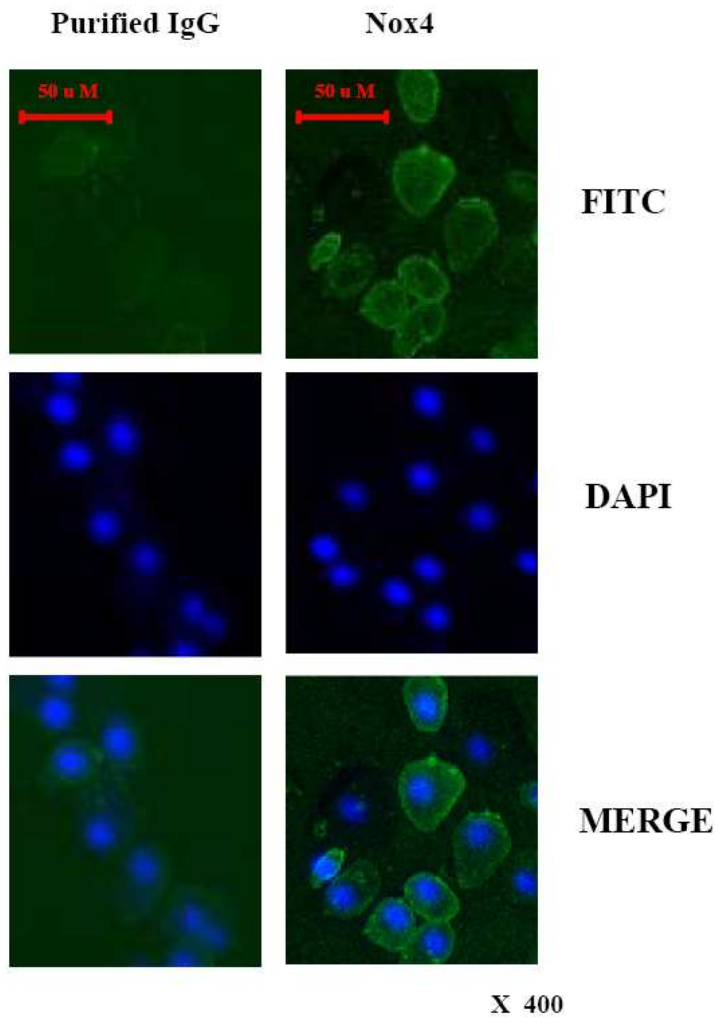


Figure 8. The localization of Nox4 in NHNE cells. The cellular localization of Nox4 proteins in NHNE was analyzed by immunofluorescence study with anti-Nox4 polyclonal antibody. Positive staining with anti-Nox4 antibody was noted in the cell membrane and part of cytoplasm of NHNE cells (right panels). No staining was detected when the primary Nox4 antibody was omitted and replaced by purified IgG (left panels).

IV. DISCUSSION

In the present study, we showed that exogenous H_2O_2 induced intracellular H_2O_2 generation in NHNE cells and that *MUC5AC* gene expression was induced by intracellular H_2O_2. Oxidative stress has detrimental effects on the host itself, since the molecules involved are potentially toxic to host cells, and the effect on host tissue may manifest as inflammatory, allergic or autoimmune diseases [29]. It has been generally understood that endogenous ROS may activate some signal pathways, affecting diverse kinds of genes in response to growth factors or inflammatory cytokines [30-32]. However, airway epithelium is continuously exposed to exogenous oxidants, including ozone, nitrogen dioxide, diesel exhaust, and cigarette smoke. Therefore, exogenous ROS may induce cellular damage or molecular changes in the airway epithelium and contribute to pathogenesis in chronic inflammatory airway disorders [22, 33, 34]. We addressed questions about the relationship between exogenous ROS and secreted mucin genes in NHNE cells. We found that exogenous H_2O_2 specifically increased only *MUC5AC* mRNA expression in a dose and time-dependent manner in NHNE cells. Most secretory proteins are located in the goblet cell cytoplasm and secreted through exocytosis. These results suggest that H_2O_2 stimulation induces the expression of *MUC5AC* mucoprotein in goblet cells, while *MUC5B, MUC6, MUC7,* and *MUC19* mucoproteins may not be affected by H_2O_2 stimulation.

ROS-induced signaling has been established in airway epithelial cells [14, 33-38]. The intracellular signal pathways responsible for the response to exogenous H_2O_2 have been evaluated in relation to EGFR and ERK1/2 MAP kinase in NCI-H292 cells [14, 33]. In particular, in normal human bronchial epithelial cells, it has been suggested that the activation of EGFR is necessary for exogenous H_2O_2 signal transduction [33, 35]. Including direct activation by its ligands, various other mechanisms may activate EGFR, such as ligand-dependent transactivation and ligand-independent activation. Ligand-dependent transactivation of EGFR is related to metalloproteinase-induced cleavage of membrane-anchored EGFR ligands and metalloproteinase inhibitors can suppress the cleavage of transmembrane ligands and transactivation of EGFR. Smoke or bacterial toxin may stimulate the cleavage or shedding of ligands in NCI-H292 cells [39]. Ligand-independent activation of EGFR can be induced directly by exposure to ROS and by different oxidative stress-inducing agents, such as ultraviolet radiation in normal human keratinocytes [40]. In our NHNE cell experiments, exogenous H_2O_2 increased the phosphorylation of EGFR. These results parallel those of studies using NCI-H292 cells and normal human bronchial epithelial cells. We also found that galardin (GM6001), a broad-spectrum matrix metalloproteinase inhibitor, did not inhibit the exogenous H_2O_2-induced phosphorylation of EGFR in NHNE cells. These findings indicate that exogenous H_2O_2 participates in the ligand-independent activation of EGFR and implicate H_2O_2

as a biologic stimulator in EGFR activation and regulation of the downstream signaling cascades, especially ERK1 MAP kinase and *MUC5AC* gene overexpression in NHNE cells.

The signal pathways between exogenous or environmental H_2O_2 and the generation of intracellular H_2O_2 have not been fully evaluated in airway epithelial cells. In our results, stimulation with exogenous H_2O_2 for 60 min increased the generation of intracellular H_2O_2 through the activation of Nox, and EGFR/ERK1 MAP kinase signal transduction mediated the Nox-induced intracellular H_2O_2 generation in NHNE cells. In other words, exogenous H_2O_2-induced *MUC5AC* gene expression may be associated with intracellular ROS generation through the EGFR/ERK1 MAP kinase signal pathway in NHNE cells.

The activity of Nox is significantly increased by various specific stimuli, including G-protein coupled receptor agonist, cytokines, growth factors, metabolic factors, hypoxia-reoxygenation and mechanical stimuli [16, 17]. Nox homolog expression has diverse in cell-specific associations. Nox1 is usually expressed in the epithelium of the gastrointestinal tract, while Nox2 is expressed in endothelial cells, including cardiomyocytes and fibroblasts. Nox3 is usually expressed in fetal tissue and Nox4 and Nox5 has been identified in the kidney, uterus, testis, vascular endothelium and fetal tissue [41].

It has been suggested that Duox is an important source of regulated H_2O_2 production in the respiratory tract [33, 42-46]. Duox1 can be activated by PMA or neutrophil elastase, producing ROS and resulting in *MUC5AC* mucin overproduction in human bronchial epithelial cells [29]. In addition, differential cytokine regulations of Duox1 and Duox2 have distinct functions in airway epithelial host defense through the production of ROS in human primary tracheobronchial epithelial cells [46].

Intracellular H_2O_2 generation of Nox4 has been characterized in vascular endothelial cells and smooth muscle cells of the heart [47, 48]. Nox4-derived ROS induce inflammatory signaling in response to LPS in human aortic endothelial cells [47] and Nox4 activates arachidonic acid through H_2O_2 generation in cardiac fibroblasts [49].

Interestingly, we found that, along with the increase of intracellular H_2O_2 generation, gene expression of Nox4 alone was increased after treatment with exogenous H_2O_2 in NHNE cells. Nox1, Nox2, Duox1 and Duox2 expressions were unchanged. Treatment with exogenous H_2O_2 also increased the expression of Nox4 protein. Furthermore, the specific inhibition of Nox4 resulted in a significant reduction in intracellular H_2O_2 generation and *MUC5AC* gene overexpression in NHNE cells. These results point to a key role for in the generation of intracellular H_2O_2 and consequently, *MUC5AC* gene expression in NHNE cells. In contrast with

other reports [42-46] stating the importance of Duox1 or Duox2 in respiratory epithelial cells, we found that Nox4 was essential for generating intracellular H_2O_2 in NHNE cells. This discrepancy may be due to differences in stimulants among reports or the use of upper versus lower airway epithelial cells.

Until now, all Nox isoforms were predicted to have transmembrane domains and has been identified in the cell membrane of various cells. These Nox families can generate ROS and the production of ROS is significantly augmented by various specific stimuli [41]. The intracellular localization of Nox4 protein, however, has been reported to be different. Sturrock et al reported that Nox4 protein expression was higher in ER and perinuclear regions of the human airway smooth muscle cells after stimulation with TGF-ß1 [28]. Pedruzzi et al also reported that treatment with 7-ketocholesterol induced Nox4 protein expression in both paranuclear and nuclear regions of aortic smooth muscle cells [27]. In our study, we found that Nox4 was localized predominantly in the cell membrane and a part of the cytoplasm of NHNE cells. The location of Nox4 expression may vary in cell and tissue-specific ways. We speculate, however, that Nox4 is located constitutively in the cell membrane, and exposure to various stimuli may increase expression of Nox4 protein in ER or ribosomes in the cytoplasm. Nox4 protein may be detected in the cell membrane of unstimulated cells and in ER or perinuclear region of cells after exposure to various stimuli.

In summary, exogenous H_2O_2 induces intracellular H_2O_2 generation via a signal pathway involving EGFR-ERK1 MAP kinase and Nox4, resulting in *MUC5AC* gene expression in NHNE cells. Nox4, one subunit of the non-phagocytic Nox system, is located in the cell membrane of NHNE cells and plays a key role in intracellular H_2O_2 generation in NHNE cells.

V. CONCLUSION

In this thesis, we proposed to identify which secreted mucin genes are induced by exogenous hydrogen peroxide (H_2O_2) and the mechanism by which these genes are up-regulated in normal human nasal epithelial (NHNE) cells. After stimulation with exogenous H_2O_2, gene expression of *MUC5AC* increased in dose and time-dependent manner, but *MUC5B, MUC6, MUC7* and *MUC19* did not. Exogenous H_2O_2 induces the intracellular H_2O_2 generation via a signal pathway involving ligand-independent activation of epidermal growth factor receptors (EGFR) and subsequent activation of ERK1 MAP kinase, Nox4 resulting in *MUC5AC* gene expression in NHNE cells. Nox4, a subtype of non-phagocytic NADPH oxidase, is located in the cell membrane of NHNE cells and play a key role in intracellular H_2O_2 generation and exogenous H_2O_2–induced *MUC5AC* gene expression in NHNE cells. Result of this thesis will be contributed for fundamental information to understand mucin hypersecretion mechamism and to treat inflammatory airway disease.

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Abstract (in korean)

사람 코 점막 상피세포에서 H_2O_2 매개 *MUC5AC* 과발현에 대한 Nox4 의 역할

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전주현

점액이 과분비는 만성 염증성 기도질환을 가진 환자의 가장 흔한 증상이며 *MUC5AC*는 점액 과분비에 기여하는 주요한 기도 점액이다. 우리가 일상 생활에서 쉽게 접할 수 있는 reactive oxygen species(ROS)는 기도 염증성 질환의 원인이 되며, 기도 점액의 과분비를 유도한다. 따라서 본 연구에서는 exogenous hydrogen peroxide (H_2O_2)에 의해 유도된 분비성 점액 유전자가 무엇인지 확인하고 이 유전자가 사람 코 점막 상피세포에서 어떠한 기전으로 발현되는지 알아보고자 하였다. 사람 코 점막 상피세포에 exogenous H_2O_2을 처리하였을 때 *MUC5AC* 유전자 발현이 농도와 시간에 비례하게 증가되었으며 *MUC5B, MUC6,MUC7, MUC19* 유전자는 발현되지 않았다. Exogenous H_2O_2는 ligand와 무관하게 EGFR을 활성화 시켰으며 그 다음 단계로 ERK1 MAP kinase가 활성화되어 결과적으로 intracellular H_2O_2의 발생이 유도되었다. 이렇게 발생된 intracellular H_2O_2는 *MUC5AC* 유전자의 과발현을 유도하였다. Intracellular H_2O_2 발생 과정 중 non-phagocytic NADPH oxidase의 아형인 Nox4가 관여하여 exogenous H_2O_2에 의한 *MUC5AC* 유전자 발현에 결정적인 역할을 하였다. 이 연구 결과는 exogenous H_2O_2에 의한 점액의 과분비 기전을 이해하고 이를 치료하는데 기초적인 자료가 될 것이다.

핵심이 되는 말: H_2O_2; *MUC5AC*; EGFR; NOX4