

Role of Reactive Oxygen Species in Transforming Growth Factor Beta1-Induced Alpha Smooth-Muscle Actin and Collagen Production in Nasal Polyp-Derived Fibroblasts

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Key Words

Alpha-smooth-muscle actin · Myofibroblast · Nicotinamide adenine dinucleotide phosphate oxidase · Nasal polyps · Reactive oxygen species · Transforming growth factor-beta1

Abstract

Background: Myofibroblasts are detected in nasal polyps and are involved in nasal polyp formation by inducing extracellular matrix accumulation. Reactive oxygen species (ROS) are released during the differentiation of fibroblasts to myofibroblasts. The purpose of this study was to investigate ROS production and nicotinamide adenine dinucleotide phosphate oxidase (NOX) expression in nasal polyp-derived fibroblasts (NPDFs) and to evaluate whether ROS from NOX mediates transforming growth factor (TGF)- β 1-induced production of alpha smooth-muscle actin (α -SMA) and collagen production. **Methods:** NPDFs were incubated and treated with TGF- β 1. The mRNA expression of *NOXs*, *α -SMA*, and *collagen type I and IV* was determined by reverse transcription-polymerase chain reaction, and the expression of α -SMA protein was determined by immunofluorescence microscopy. The amount of total soluble collagen production was analyzed by the SirCol assay. The ROS generation of cells was

investigated using the 2',7'-dichlorofluorescein-diacetate. The fluorescence was captured by fluorescent microscope and measured using a fluorometer. **Results:** Stimulation with TGF- β 1 increased ROS production by NPDFs compared with NPDFs not treated with TGF- β 1. Stimulation with TGF- β 1 increased the expression of *NOX4* mRNA most potently among various Nox enzymes. *siNOX4* was able to decrease the level of ROS production. Myofibroblast differentiation and the production of collagen in NPDFs were prevented by inhibition of ROS generation with diphenyliodonium, N-acetylcysteine, ebselen, and *siNox4*. **Conclusions:** This study showed that NOX4 and ROS have a role in myofibroblast differentiation and collagen production of TGF- β 1-induced NPDFs and that these processes are inhibited by the elimination of ROS.

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Introduction

Nasal polyps are a chronic inflammatory condition of the paranasal sinuses, the overall prevalence of which ranges from 1–4% in the general population [1]. They cause considerable morbidity, such as nasal obstruction,

rhinorrhea and anosmia. In spite of considerable efforts to identify the important factors underlying nasal polyp formation, their pathogenesis has not been fully elucidated. Among the many observed phenomena related to their formation, extracellular matrix (ECM) accumulation has been proposed as one of the important factors based on animal studies [2, 3]. It has also been shown that the number of myofibroblasts observed in nasal polyps and myofibroblast accumulation is induced by transforming growth factor (TGF)- β 1 [4].

A recent study has shown that reactive oxygen species (ROS) are released during the phenotypic transformation of fibroblasts into myofibroblasts and are important in the control of collagen deposition [5]. It has also been reported that fibroblasts produce ROS in response to growth factors, such as TGF- β 1, in the lung [6].

Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is a multi-subunit enzyme that generates superoxide by transferring electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen. NOX2 is the prototype catalytic subunit, and was originally discovered in neutrophils. There are multiple homologs of NOX2 in nonphagocytic cells, including NOX1, NOX3, NOX4 and NOX5; the homologs of NOX2 have a wide tissue distribution [7]. In phagocytes NOX-derived ROS plays an antibacterial role, whereas in nonphagocytic cells NOX-derived ROS are required for the cellular response to growth factors and cytokines [8]. It has been reported that fibroblasts produce ROS via NOX in response to growth factors, such as TGF- β 1 [9–13]. However, the role of ROS on α smooth-muscle actin (α -SMA) expression and collagen production in nasal polyp-derived fibroblasts (NPDFs) has not been reported. The purpose of this study was to investigate NOX expression in NPDFs and to evaluate whether or not ROS from NOX mediates the TGF- β 1-induced production of α -SMA and collagen production.

Methods

Reagents

Human recombinant TGF- β 1 was obtained from R&D Systems (Minneapolis, Minn., USA). N-acetylcysteine (NAC), diphenyliodonium (DPI) and ebselen (EB) were purchased from Sigma (St. Louis, Mo., USA).

Isolation of Fibroblasts and Cell Culture

To induce fibroblasts from nasal polyps, 6 patients (3 females and 3 males; mean age, 36.2 ± 8.4 years) were recruited from the Department of Otorhinolaryngology of Korea University. None of these patients had a history of nasal allergies, asthma or aspirin

sensitivity. All patients were nonsmokers and stopped taking oral and topical corticosteroids and antibiotics for at least 4 weeks before surgery. Nasal polyps were obtained from patients undergoing endoscopic sinus surgery for chronic polypoid rhinosinusitis. Before the tissue specimens were obtained, the protocols and informed consent form were approved by the Institutional Review Board for Human Research at our institution.

NPDFs were isolated from surgical tissues by enzymatic digestion with collagenase (500 U/ml; Sigma), hyaluronidase (30 U/ml; Sigma), and DNase (10 U/ml; Sigma). Following a 2-hour incubation in 5% CO₂ at 37°C in a culture plate, the cells were collected by centrifugation, washed twice, and resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, N.Y., USA) containing 10% (v/v) heat-inactivated FBS, antibiotics, 2-glutamate (Invitrogen), 100 μ g/ml of penicillin and 100 μ g/ml of streptomycin (Invitrogen). Cells were allowed to attach to the culture plate for 4 days. Nonadherent cells were removed by changing the medium. Fibroblasts were detached with EDTA solution (Invitrogen). After washing the cells, they were resuspended in medium and used for subsequent experiments. Fibroblast purity was >99% and was used for NPDFs. Cells were used at passage 4 or 5.

Cytotoxicity Assay

The cytotoxic action of NAC (0.1–3 mM), DPI (2 μ M) and EB (10 μ M) was evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method. In a 96-well microplate, NPDFs (5×10^3 cells/well) were cultivated in DMEM supplemented as described above. NAC, DPI and EB were evaluated at the above concentrations for 24 h at 37°C in an atmosphere of 5% CO₂ and 95% humidity. The cells were then incubated with MTT (5 ml; Sigma) for 4 h and the reaction was interrupted by the addition of acidified isopropanol. A microplate reader (iMark; Bio-Rad, Hercules, Calif., USA) was used to obtain the results at 570 nm. All assays were performed in triplicate.

Reverse Transcription-Polymerase Chain Reaction

NPDFs (5×10^6 cells/ml) were pretreated with or without NAC (0.1–3 mM), DPI (2 μ M), or EB (10 μ M) for 2 h before 24 h TGF- β 1 stimulation. NAC and EB are antioxidant and DPI is an inhibitor of NOX. Total RNA was extracted using a commercially available reagent (TRIzol; Takara, Japan). Total RNA from each sample was reverse-transcribed in 20 μ l of reaction mixture containing 2.5 U of Moloney murine leukemia virus reverse transcription transcriptase (RT; Promega, Madison, Wisc., USA) and 50 pmol of dNTP at 37°C for 60 min. Oligonucleotide primers for PCR were synthesized at Bioneer Corp. (Daejeon, South Korea) (table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to verify that equal amounts of RNA were used for RT and polymerase chain reaction (PCR) amplification from different experimental conditions. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The gels were certificated using a Molecular Imager Chemi-Doc XRS+ (Bio-Rad, Hercules, Calif., USA).

Transfection with Small Interference RNA

For the NOX4 RNA interference, an siRNA specific for human NOX4 (siNOX sense, 5'-ACU GAG GUA CAG CUG GAU GUU-3' and antisense, 3'-CAU CCA GCU GUA CCU CAG UUU) was employed. The universal negative control siRNA (Invitrogen)

Table 1. Primer sequences used for RT-PCR

Primer		Sequence	Size
NOX1	Sense	5'-CCA GGA TTG AAG TGG ATG GT-3'	306 bp
	Antisense	5'-CGG TGA GGA AGA GAC CGT AG-3'	
NOX2	Sense	5'-GGA GTT TCA AGA TGC GTG GAA ACT A-3'	549 bp
	Antisense	5'-GCC AGA CTC AGA GTT GGA GAT GCT-3'	
NOX3	Sense	5'-CCA TGG GAC GGG TCG GAT TGT-3'	376 bp
	Antisense	5'-GGG GGC AGA GGT AAG GGT GAA GG-3'	
NOX4	Sense	5'-CTG GAG GAG CTG GCT CGC CAA CGA AG-3'	516 bp
	Antisense	5'-GTG ATC ATG AGG AAT AGC ACC ACC ATG CAG-3'	
NOX5	Sense	5'-ATC AAG CGG CCC CCT TTT TTC AC-3'	238 bp
	Antisense	5'-CTC ATT GTC ACA CTC CTC GAC AGC-3'	
α -SMA	Sense	5'-GGT GCT GTC TCT CTA TGC CTC TGG A-3'	321 bp
	Antisense	5'-CCC ATC AGG CAA CTC GAT ACT CTT C-3'	
Collagen type I	Sense	5'-GTC TTC CTG GCC CCT CTG GTG-3'	391 bp
	Antisense	5'-TCG CCC TGT TCG CCT GTC TCA-3'	
Collagen type IV	Sense	5'-CCA GGA CCA AGT GGA AGA G-3'	318 bp
	Antisense	5'-CTG CAA CAC CAT CTC TGC C-3'	
GAPDH	Sense	5'-GTG GAT ATT GTT GCC ATC AAT GAC C-3'	270 bp
	Antisense	5'-GCC CCA GCC TTC TTC ATG GTG GT-3'	

served as a control. Individual siRNAs (100 nM), lipofectamine and opti-MEM medium were mixed and incubated at room temperature for 30 min. siRNA-lipofectamine complexes were added to the cells and removed after 24 h. The cells were washed and placed in serum-free medium for 24 h, and were then treated with or without TGF- β 1 for the indicated time and harvested for RNA extraction.

Measurement of ROS Generation

The ROS generation of cells was investigated using 2',7'-dichlorofluorescein-diacetate (DCFH-DA; Molecular Probes Inc., Eugene, Oreg., USA). NPDPs were pretreated with or without NAC (3 mM), DPI (2 μ M) or EB (10 μ M). After TGF- β 1 treatment, DCFH-DA (10 μ M) was added for 30 min. The medium was removed and the cells were washed 3 times with serum-free medium. The fluorescence was measured at 535 nm with an excitation wavelength of 485 nm using a fluorometer (Beckman Coulter, Fullerton, Calif., USA). The cells were then examined under a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Immunofluorescence staining of α -SMA Protein

NPDPs (2×10^3 cells/ml) were plated on coverslips. Fibroblasts were pretreated with NAC (3 mM), DPI (2 μ M) and EB (10 μ M) for 2 h before TGF- β 1 stimulation for 24 h. The cells were then fixed in PBS containing 4% paraformaldehyde for 30 min, blocked with 3% bovine serum albumin, incubated with an anti- α -SMA antibody (1: 200) for 2 h and washed 3 times with PBS for 5 min. They were then incubated in goat anti-mouse IgG Alexa

Fluor 488 (Invitrogen) at a dilution of 1:200 for 1 h and washed 3 times with PBS for 5 min. They were mounted in Vectashield (Vector Laboratories Inc., Burlingame, Calif., USA) with 4',6'-diamidino-2-phenylindole and then visualized on a fluorescence microscope under identical settings.

Collagen Measurements

Total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay (Biocolor, Belfast, UK). For these experiments, confluent cells in culture dishes (25 cm²) were incubated for 24 h with 1 ml of DMEM-5% FBS. One milliliter of Sirius red dye, an anionic dye that reacts specifically with basic side-chain groups of collagens under assay conditions, was added to 400 μ l of supernatant and incubated with gentle rotation for 30 min at room temperature. After centrifugation at 12,000 g for 10 min, the collagen-bound dye was redissolved with 1 ml of 0.5 M NaOH, and absorbance at 540 nm was measured by an enzyme-linked immunosorbent assay (MRX; Dynex, Chantilly, Va., USA). The absorbance was directly proportional to the amount of newly formed collagen in the cell culture supernatant.

Statistical Analysis

The statistical significance of the difference between the control and experimental data was analyzed using Wilcoxon's signed-rank test. A p value <0.05 was accepted as statistically significant.

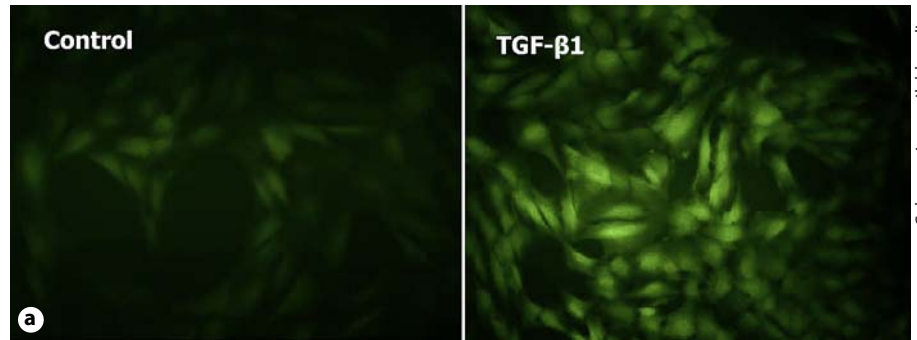
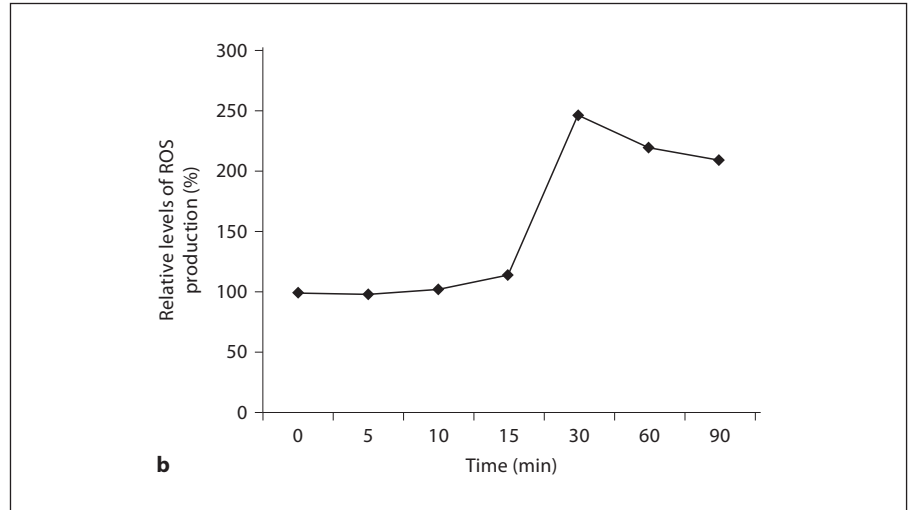


Fig. 1. Effect of TGF- β 1 on ROS production in NPDPs. ROS production was also determined by fluorescent microscopy using a fluorescent probe (2',7'-dichlorofluorescein diacetate). **a** Fluorescent microscopic finding representative of 6 independent experiments. $\times 200$. **b** ROS generation of cells was investigated using a fluorometer.



Results

The Cytotoxicity of NAC, DPI and EB

We examined the cytotoxicity of NAC (0.1–3 mM), DPI (2 μ M) and EB (10 μ M) on NPDPs using an MTT assay. They did not have a cytotoxic effect for 48 h (data not shown).

Effect of TGF- β 1 on ROS Generation in NPDPs

We examined whether TGF- β 1 stimulates ROS production in NPDPs. The effect of TGF- β 1 on ROS production was observed by fluorescent microscopy (fig. 1a). TGF- β 1-stimulation increased ROS production compared to no TGF- β 1 treatment. Continuous evaluation was made using a fluorometer; after an initial stimulation, ROS production increased after 10 min and reached a maximum at 30 min (fig. 1b).

mRNA Expression of NOX in TGF- β 1-Induced NPDP

Since TGF- β 1 increased ROS production in NPDPs, we hypothesized that the increased ROS production in

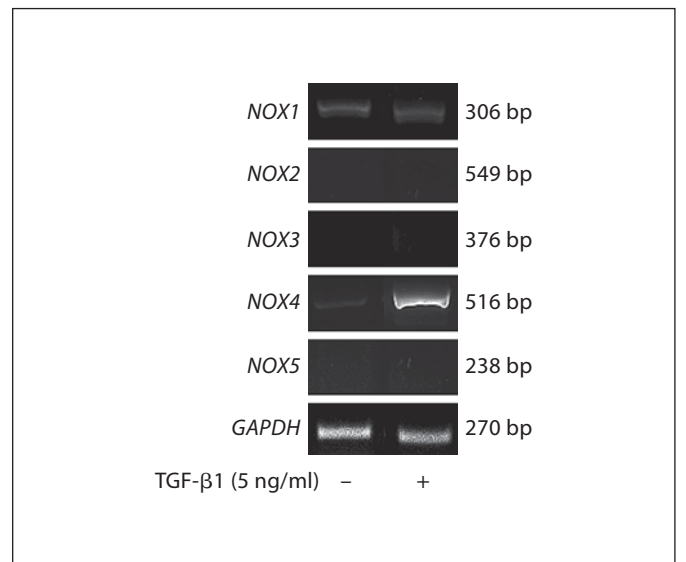


Fig. 2. mRNA expression of NOX in TGF- β 1-stimulated NPDPs analyzed by RT-PCR.

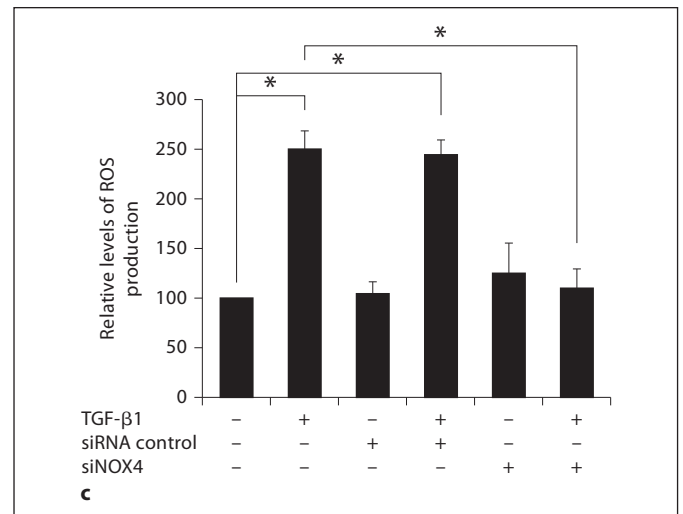
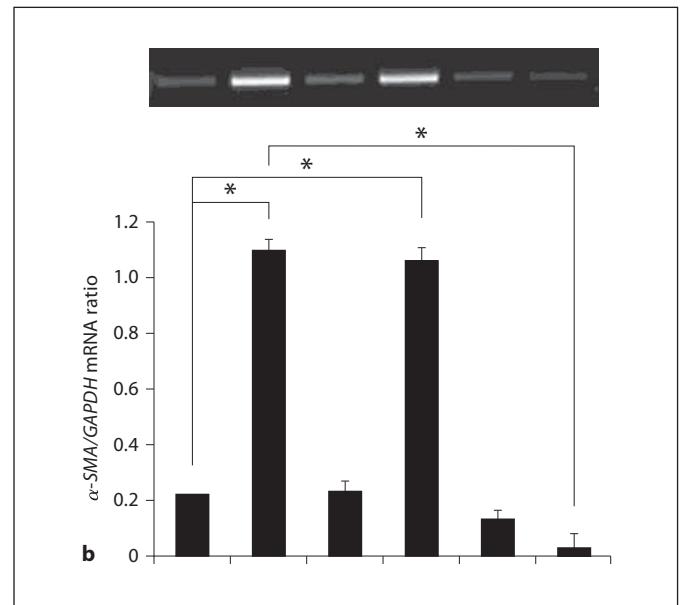
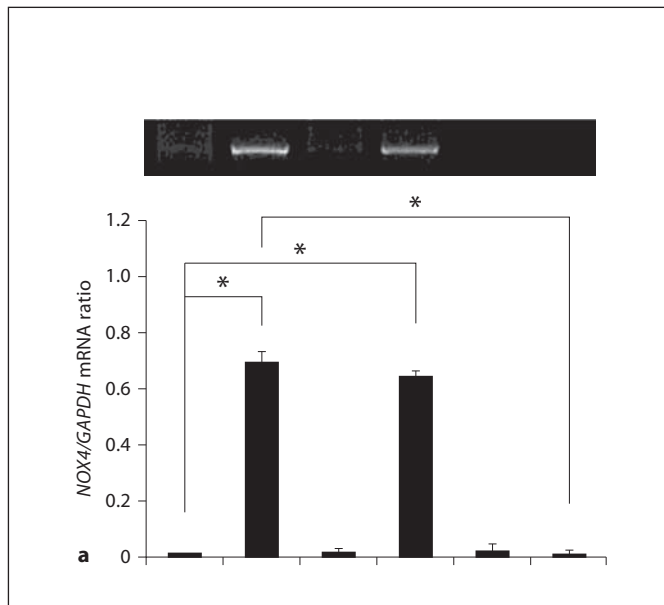


Fig. 3. The role of NOX4 on α -SMA expression in TGF- β 1-induced NPDFs. **a** NOX4 mRNA was semi-quantified by RT-PCR. **b** α -SMA mRNA was semi-quantified by RT-PCR. **c** The production of ROS was measured by fluorometer using a fluorescent probe (2',7'-dichlorofluorescein diacetate). The mean $\pm$ SE of data from 6 experiments are shown. * $p < 0.05$.

NPDFs is mediated by increased expression of a NOX. To identify which NOX is upregulated, we stimulated NPDFs with TGF- β 1 and evaluated the expression of various NOX enzymes at the level of RNA. After stimulation with TGF- β 1, NOX4 mRNA was significantly upregulated (fig. 2).

Role of NOX on α -SMA Expression in TGF- β 1-Induced NPDFs

Additional studies were performed to determine whether or not NOX4 is necessary for α -SMA expression in TGF- β 1-induced NPDFs. We previously showed that TGF- β 1-stimulation increased the expression of α -SMA

[14]. We downregulated the expression of NOX4 mRNA using transfection with small interference oligonucleotide RNA directed against NOX4 (siNOX4). First, we confirmed that siNOX4 is able to decrease the level of NOX4 mRNA (fig. 3a). On transfection with siNOX4, the TGF- β 1-induced expression of NOX4 mRNA was reduced. To assess whether or not NOX4 is required for α -SMA expression, we semiquantified α -SMA at the mRNA level after transfection of cells with siNOX4. The α -SMA mRNA was dramatically reduced by the siRNA against NOX4 (fig 3b). ROS production was evaluated continuously using a fluorometer. siNOX4 was able to decrease the level of ROS production (fig. 3c).

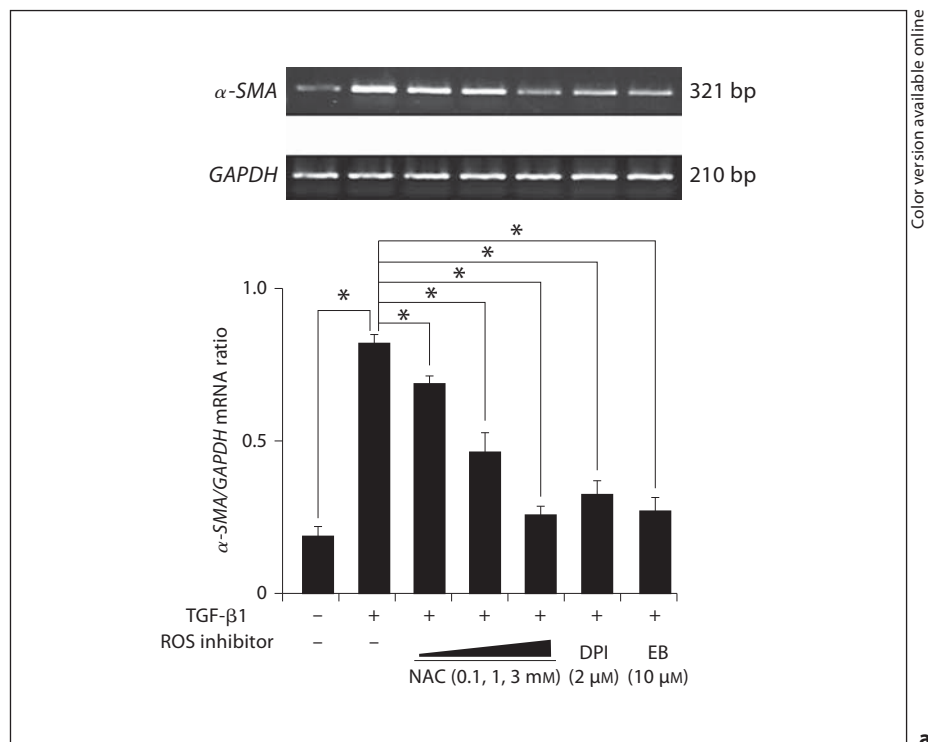
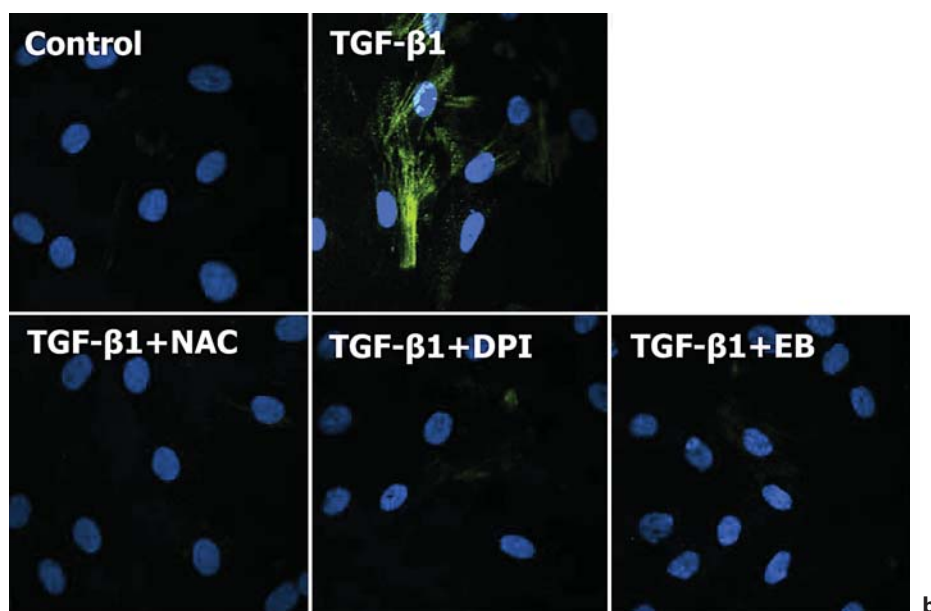
**a****b**

Fig. 4. **a** Effects of ROS inhibition on the significantly increased expression of α -SMA mRNA in TGF- β 1-induced NPDFs, as determined by RT-PCR and densitometric analysis. The mean $\pm$ SE of data from 6 experiments are shown. * $p < 0.05$ compared to TGF- β 1 alone. **b** Effects of ROS inhibition on the expression of α -SMA protein in TGF- β 1-induced NPDFs, as determined by fluorescence staining. The nucleus was counterstained using DAPI (blue). $\times 400$. Representative of 6 independent experiments.

Effect of NAC, DPI and EB on α -SMA Expression in TGF- β 1-Induced NPDFs

Cells were pretreated with NAC, DPI and EB for 2 h and stimulated with TGF- β 1 for 24 h. The level of expression of α -SMA mRNA was determined by RT-PCR. TGF- β 1 significantly increased the level of expression of α -

SMA mRNA. Pretreatment with NAC, DPI and EB significantly inhibited TGF- β 1-induced α -SMA mRNA expression (fig. 4a). In immunofluorescence staining, the addition of NAC, DPI and EB decreased the number of α -SMA protein-positive cells (fig. 4b).

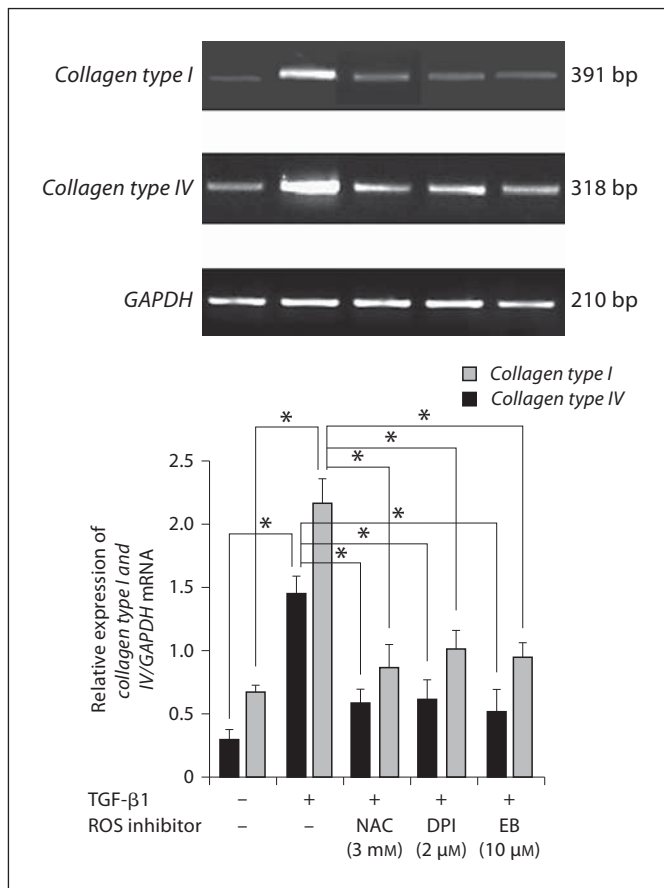


Fig. 5. Effects of ROS inhibition on the significantly increased expression of *collagen type I* and *IV* mRNA in TGF-β1-induced NPDFs, as determined by RT-PCR and densitometric analysis. The mean $\pm$ SE of data from 6 experiments are shown. * $p < 0.05$.

Effect of NAC, DPI and EB on Collagen Production in TGF-β1-Induced NPDFs

To determine the inhibitory effect of NAC, DPI and EB on TGF-β1-induced *collagen type I* and *IV* mRNA expression, we pretreated cells with NAC, DPI and EB for 2 h before stimulating the cells with TGF-β1 for 24 h. The expression of *collagen type I* and *IV* mRNA was increased by stimulation with TGF-β1 and was notably decreased by pretreatment with NAC, DPI, and EB (fig. 5).

To determine the inhibitory effect of NAC, DPI and EB in TGF-β1-induced soluble collagen production, we pretreated cells with NAC, DPI and EB and stimulated the cells with TGF-β1. The addition of NAC, DPI and EB noticeably reduced TGF-β1-induced soluble collagen production (fig. 6).

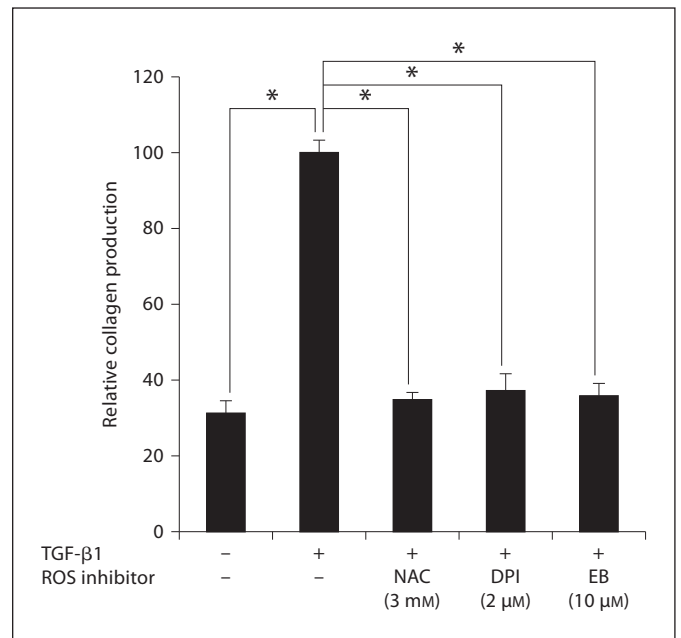


Fig. 6. Effects of ROS elimination on the significantly increased production of total soluble collagen in TGF-β1-induced NPDFs. Total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay. The mean $\pm$ SE of data from 6 experiments are shown. * $p < 0.05$.

Discussion

In this study, we showed that fibroblasts produce ROS in response to TGF-β1 and that ROS from fibroblasts are involved in TGF-β1-induced expression of α -SMA and collagen production. Inhibitors of NOX and ROS scavengers, such as DPI, NAC and EB significantly inhibited TGF-β1-induced α -SMA mRNA and protein expression. TGF-β1 enhances the expression of NOX4 homologs in the NOX family. In addition, NOX4 seems to be involved in the phenotype change of NPDFs because of the ability of siRNA to reduce TGF-β1-induced expression of α -SMA mRNA. Taken together, we provide evidence that NOX4-derived ROS plays a key role in TGF-β1-induced myofibroblast differentiation and collagen production in NPDFs.

Accumulation of ECM is an important process in the characteristic structural modification of nasal polyps [15]. Myofibroblasts, which are found in nasal polyps, appear to be involved in the above process. They are the activated cell phenotype of fibroblasts and have a high capacity for ECM protein secretion. Fibroblast transformation into myofibroblasts is associated with enhanced

matrix production. In nasal polyps, growth factors, such as TGF- β 1, stimulate fibroblast proliferation and myofibroblast differentiation [16]. In our previous study, we showed that TGF- β 1 significantly increased the expression of α -SMA as well as the production of collagen in NPDFs [14].

NOX is a membrane-associated oxidase that is found in a variety of cell types, including neutrophils, vascular smooth muscle cells, endothelial cells, cardiac myocytes and vascular, cardiac and lung fibroblasts. NOXs generate superoxide by transferring electrons from NADPH to molecular oxygen. Although the prototype catalytic subunit NOX2 was originally discovered in neutrophils, there are multiple homologs including NOX1, NOX3, NOX4 and NOX5 in nonphagocytic cells with a wide tissue distribution. Phagocytic NOXs release large bursts of ROS extracellularly that are used for host defense against bacterial and fungal pathogens, whereas nonphagocytic NOXs release lower levels of ROS initiating cellular signaling events that control a variety of cellular functions, including proliferation, hypertrophy, apoptosis, cytokine production and the expression of matrix regulatory proteins [9]. NOX4 was originally identified as the NOX homolog highly expressed in the kidney and is also found in osteoclasts, endothelial cells, smooth-muscle cells, hematopoietic stem cells, neurons and fibroblasts. It is known that upregulation of NOX4 is observed in response to endoplasmic reticulum stress, shear stress, hypoxia and TGF- β 1. In this study, we stimulated NPDFs with TGF- β 1 and found that NOX4 was the only NOX homolog that was significantly upregulated [17].

In several studies, intimate connections between ROS and the phenotypic transformation of fibroblasts to myo-

fibroblasts were observed. It was first shown in cardiac fibroblasts that NOX plays a role in TGF- β 1-induced fibroblast differentiation to myofibroblasts [10]. Indeed, the authors showed that NOX4 mediates TGF- β 1 NADPH-driven superoxide production and is required for the expression of α -SMA, fibronectin, collagen and connective tissue growth factor. In the studies involving kidney and pulmonary fibroblasts, the phenotypic transformation of fibroblasts into myofibroblasts, as characterized by the expression of α -SMA, is regulated through NOX-generated ROS [11, 12]. We also showed that NOX4 plays a role in TGF- β 1-induced phenotypic transformation into myofibroblasts. However, more study is needed to identify the mechanisms by which TGF- β 1 increases the expression of NOX4 in NPDFs.

This study showed that NOX4 and ROS play a role in myofibroblast differentiation and collagen production of TGF- β 1-induced NPDFs and that the elimination of ROS inhibits TGF- β 1-induced myofibroblast differentiation and collagen production in NPDFs. The frequent recurrence of nasal polyps after endoscopic sinus surgery requires additional therapeutic modalities. The results of our study suggest antioxidants are a possible candidate for the inhibition of nasal polyp formation. Further testing in patients will be required to verify this finding.

Acknowledgement

This study was supported by a grant from the Korea Healthcare Technology R&D Projects, Minister for Health, Welfare & Family Affairs, Republic of Korea (A090084).

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