

Transforming Growth Factor β 1-Induced NADPH Oxidase-4 Expression and Fibrotic Response in Conjunctival Fibroblasts

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PURPOSE. Fibrotic scarring after ocular surgeries and chemical burn injuries can impede clarity of the cornea and cause vision impairment. Transforming growth factor β (TGF β) signaling pathway is known to mediate fibrotic scarring, and NADPH oxidase-derived reactive oxygen species has been shown to be an effector molecule that facilitates TGF β 1-mediated responses. The present study explores the expression profile and functional importance of NADPH oxidase (Nox) in conjunctival fibroblasts. In addition, the effect of curcumin on the TGF β 1-induced NADPH oxidase expression and collagen synthesis was also investigated.

METHODS. The mRNA expression of Nox isoforms in rabbit conjunctival fibroblasts was measured by real-time PCR. The production of hydrogen peroxide (H₂O₂) and total collagen by these cells was measured by Amplex Red assay and Picro-Sirius red assay, respectively. Nox4 was knocked down by adenovirus-mediated siRNA targeting Nox4 (Adv-Nox4i).

RESULTS. We describe for the first time that conjunctival fibroblasts express mRNA encoding for Nox2, Nox3, Nox4, and Nox5. TGF β 1 was found to induce Nox4 mRNA expression and total collagen release by these cells ($P < 0.05$; $n = 4$), and both responses are blocked by Smad3 inhibitor SIS3. Suppressing Nox4 gene transcription with Adv-Nox4i completely attenuated TGF β 1-stimulated H₂O₂ release and collagen production by conjunctival fibroblasts ($P < 0.05$; $n = 4-6$). Similarly, curcumin also inhibited TGF β 1-induced Smad3 phosphorylation, Nox4-derived H₂O₂ production, and total collagen synthesis by conjunctival fibroblasts ($P < 0.05$; $n = 4-6$).

CONCLUSIONS. The present study suggests that TGF β 1-mediated production of collagen by conjunctival fibroblasts involves Nox4-derived H₂O₂ pathway and this effect of Nox4 is abrogated by curcumin. This mechanism might be exploited to prevent fibrotic scarring after surgeries and chemical burn injuries in the eye.

Keywords: conjunctiva, Nox4, TGF β 1, curcumin, ocular fibrosis

The cornea is a transparent tissue covering the anterior portion of the eye and is essential for vision whilst the conjunctiva covers the sclera and influences the physiological functions of the cornea. The fibrotic scarring in the cornea and conjunctiva after surgeries (e.g., glaucoma filtration surgery, pterygium and corneal refractive surgeries, lens capsule postcataract surgery) or injuries (e.g., chemical burns) can lead to reduced corneal transparency and potentially impair vision.¹⁻⁴ The antiproliferative drugs mitomycin C and 5-fluorouracil are used worldwide as antiscarring treatments for a number of surgical interventions in the eye to improve postoperative outcomes. These drugs are cytotoxic in nature and can be associated with increased postoperative complications.^{2,5,6} In the case of ocular chemical burn injury, first-line treatment consists of topical steroids to reduce ocular inflammation and scar formation as well as to promote the wound-healing response.⁷⁻⁹ Topical corticosteroids also have undesired effects such as corneal thinning and development of glaucoma.^{7,9,10} Therefore, identifying a novel target in the

fibrotic pathway in eyes might be advantageous over current therapy.

Fibrotic scarring in eyes appears to follow a similar mechanism to that seen in fibrotic disorders in other organs such as heart, kidneys, and lungs.^{2,4} In this regard, transforming growth factor β (TGF β) is known to be a key contributor to fibrotic scarring in eyes.¹¹⁻¹⁴ One of TGF β 's signaling mechanisms in the development of fibrosis involves an excess production of extracellular matrix proteins including collagens and fibronectin by fibroblasts.^{4,15,16} As such, suppressing TGF β signaling would provide a means of preventing fibrotic scarring in eyes.^{2,4,17-19} Recently, we and others have shown that an effector molecule, NADPH oxidase (Nox), that acts downstream of TGF β plays a major role in profibrotic responses in fibroblasts from hearts,^{20,21} lungs,^{22,23} and kidneys.²⁴ NADPH oxidase comprises seven isoforms and is known to cause production of reactive oxygen species (ROS).²⁵ While an expression profile of Nox isoforms has been characterized in fibroblasts from a variety of organs such as hearts,^{20,21} lungs,^{22,23} and kidneys,²⁴ the expression pattern of Nox



isoforms and its functional importance in fibroblasts derived from eyes remain elusive. In the present study, we first defined the expression of Nox isoforms Nox1 to Nox5 and their role in TGFβ-mediated profibrotic response in rabbit conjunctival fibroblasts. These cells are known to have a crucial role in fibrotic scarring seen in ocular injury.

Curcumin is known to have antioxidant and anti-inflammatory properties.²⁶ The underlying mechanism of these effects appears to be diverse but a recent study²⁷ highlights that it inhibits a key coactivator enzyme, namely, p300 histone acetyltransferase (p300 HAT). p300 HAT is essential for activation of transcription factors such as Smad and nuclear factor κB (NF-κB) that participate in the activation of fibrotic genes such as collagen and fibronectin.^{11,28} Recently, we have shown that inhibition of p300 HAT reduces both expression and activity of Nox4 in endothelial cells.²⁹ In the present study, we also investigated the effect of curcumin on the expression of Nox isoforms, ROS generation, and total collagen production in the absence and presence of TGFβ1 by rabbit conjunctival fibroblasts.

MATERIALS AND METHODS

Conjunctival Fibroblast Culture

The experiment adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Conjunctival fibroblasts were isolated from New Zealand white rabbits by mincing the tissue, then digesting in serum-free Roswell Park Memorial Institute medium 1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) containing collagenase I (1 mg/mL; Worthington Biochemical Corporation, Lakewood, NJ, USA) and then in trypsin (0.05%)-EDTA (0.02%; Lonza, City, Switzerland) at 37°C. Supernatant containing conjunctival fibroblasts was transferred to RPMI supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Corp., St. Louis, MO, USA), penicillin (100 U/mL; Invitrogen), and streptomycin (100 µg/mL; Invitrogen) every 20 to 25 minutes, and conjunctival fibroblasts collected from three digestive cycles were pelleted, resuspended in fresh medium, and cultured at 37°C in 5% CO₂. Rabbit conjunctival fibroblasts were immortalized by using SV40 (simian virus 40) T antigen. Cells from passages 5 to 20 were used. Cells originated from conjunctival tissue and maintained morphology typical of conjunctival fibroblasts throughout. These cells were used to assess the expression of Nox isoforms and production of hydrogen peroxide (H₂O₂) and collagen.

Experimental Setup

Unless otherwise specified, the cells were serum starved overnight and treated with TGFβ1 (5 ng/mL; Sigma-Aldrich Corp.) for 6 hours for H₂O₂ measurement and 24 hours for total collagen assay. In some cases, the cells were pretreated with Smad3 inhibitor SIS3 (5 µM; Sigma-Aldrich Corp.), superoxide dismutase (SOD)/catalase mimetic EUK-134 (25 µM; Cayman Chemical, Ann Arbor, MI, USA) or curcumin (10 µM; Sigma-Aldrich Corp.) for 30 minutes before addition of TGFβ1 (5 ng/mL). SIS3, EUK-134, and curcumin were dissolved in 100% dimethyl sulfoxide (DMSO). The final concentration of DMSO was maintained at 0.1% in all experiments. The effect of SIS3, EUK-134, and curcumin on cell loss was tested after 24 hours by using total protein measurement using Bradford assay (Supplementary Fig. S1).

Adenovirus Infection

We silenced Nox4 gene expression by using adenoviral vectors expressing small interfering RNA (siRNA) targeting human

Nox4 nucleotides 418 to 436 from the start codon (Adv-Nox4i) as described previously.³⁰ Adenovirus expressing scrambled siRNA (Adv-Ctrl RNAi) was used as a control. Cells were infected with 2000 multiplicity of infection of Adv-Ctrl RNAi or Adv-Nox4i for 24 hours in Opti-MEM medium (Life Technologies, Waltham, MA, USA) and allowed to recover in RPMI + 10% FBS medium for another 24 hours. All experiments were performed at 48 hours after infection. The effect of Adv-Ctrl RNAi and Adv-Nox4i on cell loss was tested after 48 hours by using total protein measurement using Bradford assay (Supplementary Fig. S1).

Determination of Extracellular H₂O₂

H₂O₂ levels were detected by using Amplex Red assay kit (Molecular Probes; Life Technologies) according to manufacturer's instructions. In cell-free system different concentrations of curcumin (1–100 µM) were exposed to 10 µM H₂O₂ or spontaneous enzymatic generation of H₂O₂ using xanthine (100 µM; Sigma-Aldrich Corp.)/xanthine oxidase (0.03 U/mL; Sigma-Aldrich) prepared in Krebs-Ringer bicarbonate buffer containing Amplex Red reagent (50 µM) and horseradish peroxidase (HRP; 0.1 U/mL). Fluorescence was then measured for 30 minutes with excitation and emission at 550 and 590 nm, respectively, by using a polarstar microplate reader (BMG Labtech, Ortenberg, Germany) at 37°C. Extracellular H₂O₂ from cells was also measured as described previously.³⁰ Briefly, cells (2 × 10⁴ cells/well) were seeded in a 24-well plate. Cells were treated with and without TGFβ1 (5 ng/mL) or curcumin for 24 hours in Krebs-Ringer bicarbonate buffer containing 0.1% FBS, Amplex Red reagent (50 µM), and horseradish peroxidase (HRP; 0.1 U/mL). Fluorescence was then measured as described above.

Gene Expression Analysis

Cells (1 × 10⁵ cells/well) were seeded in six-well plates. Serum-deprived cells were treated with various inhibitors or TGFβ1. Total RNA from treated cells was extracted with the TRI Reagent according to manufacturer's instructions (Ambion, Waltham, MA, USA). cDNA was prepared from 200 ng total RNA by using high-capacity-performance reverse transcription reagents (Applied Biosystems, Waltham, MA, USA) at 25°C for 10 minutes, 37°C for 2 hours, followed by 85°C for 5 seconds in a thermal cycler (BioRad-DNA Engine; Bio-Rad, Hercules, CA, USA). The quantitative real-time PCR reactions were performed in a 7500 system (Applied Biosystems, Life Technologies) by using SYBER green master mix (Applied Biosystems, Life Technologies) and designed gene-specific primer sets for Nox1 to Nox5 and β-actin (Table). Nuclease-free water was used as a negative control. The reaction specificity was confirmed by gel electrophoretic analysis of product in 1.5% agarose gel after real-time PCR (Fig. 1) and melt curve analysis. The cycle threshold (CT) values from all quantitative real-time PCR experiments were analyzed by using $\Delta\Delta$ CT method. Data were normalized to β-actin and expressed as fold changes over that in control treatment group. β-Actin has been validated as the appropriate housekeeping gene to explore the effect of TGFβ1 because there is little variation in the gene expression of β-actin in cells treated with and without TGFβ1 when β-actin expression is normalized to another housekeeping gene, namely, 18s (data not shown).

Western Blot Analysis

Cells (1 × 10⁵ cells/well) were cultured in 12-well plates and protein was extracted as previously described.³¹ Equal amounts of protein were then separated by electrophoresis

TABLE. Sequences of PCR Primer Sets Used to Amplify NADPH Oxidase Isoforms for Rabbit (*Oryctolagus cuniculus*) Conjunctival Fibroblasts

Gene Name	Gene ID	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon Size, bp
NOX1	100008852	caatctgctgtccttcctgta	catctccttccttcccatga	239
NOX2 (CYBB)	100008801	gtggagtggtgtgtaaatgc	tttggaggaggatgtgatga	219
NOX3	100358363	gtggctgggaatgaacttgt	attcaggcacagtgcagatg	133
NOX4	100339697	ttggcttttgatttctggac	tactggccaggctcttgcctt	165
NOX5	100301540	gattcctttgccctgttcgac	ccatcgacatcgtaacacctg	136
ACBT (β-actin)	100009272	tccttgagagaagagctacga	gtacagggtccttgccggatgt	180

using 8% SDS-PAGE gels and transferred to hybond polyvinylidene difluoride membrane (GE Healthcare, NSW, Australia). After blocking with 5% nonfat milk in buffer containing Tris-HCl (20 mM, pH 7.5), NaCl (100 mM), and Tween-20 (0.1%), respective membranes were incubated at 4°C overnight with primary antibodies against phospho-Smad2/Smad3 (rabbit monoclonal No. 8828, 1:1000; Cell Signaling Technology, Danvers, MA, USA), total-Smad2/3 (rabbit monoclonal No. 8685, 1:1000; Cell Signaling Technology), mouse monoclonal anti-β-actin (1:4000; Merck Millipore, Darmstadt, Germany). Proteins were detected by using an enhanced chemiluminescence detection kit (GE Healthcare) with horseradish peroxidase conjugated to appropriate secondary antibodies (Bio-Rad). The image was captured and processed by using ChemiDoc Imagers (Bio-Rad). Ratio of phosphorylated Smad3 and total Smad3 bands were calculated by using Image J software³² (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and presented as an arbitrary unit (AU).

Picro-Sirius Red Spectrophotometric Assay

Total collagen was measured by using Picro-Sirius red-based high-throughput assay as described previously.³³ In brief, cells (2.5×10^4) cultured in 96-well plates were fixed in methanol for 1 hour at -20°C, carefully washed once with PBS, and incubated in Picro-Sirius red (0.1%; Sigma-Aldrich Corp.) at room temperature for 1 hour. Picro-Sirius red was removed, and cells were washed three times with 0.1% acetic acid. Picro-Sirius red was then eluted in 0.1 N sodium hydroxide, 200 μL/well, the plates were placed on a rocking platform at room temperature for 1 hour, and the optical density at 540 nm was determined with a Bio-Tek microplate spectrophotometer (Bio-Tek, Winooski, VT, USA).

Statistical Analysis

Values are expressed as mean ± SEM. All experiments were carried out in replicates by using at least four independent cell cultures. The mean results were analyzed with 1-way analysis of variance (ANOVA) followed by post hoc Tukey analysis. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

Expression of Nox Isoforms in Conjunctival Fibroblasts

To define the isoforms of NADPH oxidase in rabbit conjunctival fibroblasts and better understand potential mechanism(s) of Nox activation by TGFβ1, we analyzed the expression of mRNA for most known isoforms of the Nox and Duox family. Real-time PCR showed that Nox isoforms Nox2, Nox3, Nox4, and Nox5 are expressed in the rabbit conjunctival fibroblasts

(Fig. 1), whereas Nox1 (Fig. 1), Duox1, and Duox2 were undetectable (data not shown).

TGFβ1 Induced Nox4 Gene Expression in Conjunctival Fibroblasts

Of all Nox isoforms identified in rat kidney fibroblast cell line²⁴ and mouse primary lung²² and cardiac fibroblasts,²⁰ Nox4 appears to be the primary isoform involved in TGFβ-induced fibrotic responses such as collagen production.^{20,22} We therefore first determined if TGFβ1 stimulates gene expression of Nox4 in conjunctival fibroblasts. Cells were treated with TGFβ1 (5 ng/mL) for 3, 6, and 24 hours, and TGFβ1 significantly increased Nox4 mRNA expression at all three time points (Fig. 2A). Importantly, TGFβ1 was also found to increase the production of H₂O₂ in parallel with the upregulation of Nox4 gene (Fig. 2B), confirming the H₂O₂ generation capacity of the enzyme. We have also determined the gene expression of other isoforms detected in rabbit conjunctival fibroblasts under the stimulation of TGFβ1. The mRNA expression of Nox2 and Nox3 did not change, whereas Nox5 expression significantly decreased after treatment with TGFβ1 for up to 24 hours (Supplementary Fig. S2). We then explored if TGFβ1 affects collagen production in conjunctival fibroblast at 3, 6, and 24 hours. Interestingly, the increase in collagen synthesis was only seen at 24 hours, which occurs after Nox4 gene upregulation and H₂O₂ production (Fig. 2C), suggesting that the stimulatory effect of TGFβ1 on collagen production may require Nox4 expression and H₂O₂ production.

TGFβ1 Signaling Is Induced via a Smad3-Dependent Pathway in Conjunctival Fibroblasts

The phosphorylation of Smad3 is known to be involved in Nox4 gene upregulation and collagen production in fibroblasts treated with TGFβ1.^{24,34} We explore if TGFβ1 induction of Nox4 mRNA expression and collagen production is also dependent on Smad3 in conjunctival fibroblasts. As expected, TGFβ1 induced phosphorylation of Smad3 within 30 minutes in these cells (Fig. 3A). Treatment with Smad3 inhibitor SIS3 was found to abolish TGFβ1-induced Nox4 mRNA expression (Fig. 3B) and total collagen production (Fig. 3C), confirming that Smad3 is required for both responses.

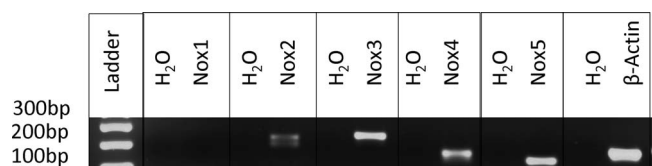


FIGURE 1. Expression profile of Nox isoforms in rabbit conjunctival fibroblasts. Total RNA obtained from rabbit conjunctival fibroblasts and the expression of Nox isoforms were examined by RT-PCR. PCR products of Nox2, Nox3, Nox4, and Nox5 and the housekeeping gene β-actin. Nuclease free water (H₂O) was used as negative control.

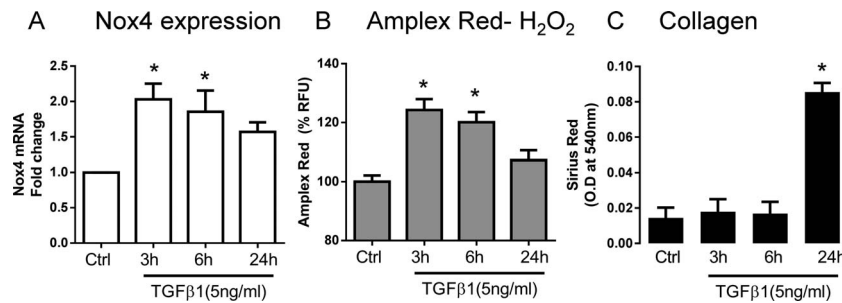


FIGURE 2. Effect of TGFβ1 on Nox4 expression and H₂O₂ formation in conjunctival fibroblasts. (A) TGFβ1 (5 ng/mL) induced Nox4 mRNA expression levels in a time-dependent manner in rabbit conjunctival fibroblasts. (B) H₂O₂ generation as detected by Amplex Red assay was increased after 3-hour and 6-hour treatment with TGFβ1 (5 ng/mL). (C) Total collagen expression was increased at 24 hours following TGFβ1 treatment in the rabbit conjunctival fibroblasts. All data are mean ± SEM from four independent experiments, **P* < 0.05 from control (Ctrl) without treatment.

Suppression of Nox4 Decreased TGFβ1-Induced H₂O₂ Generation and Collagen Production

To illustrate the functional importance of TGFβ1-induced Nox4 expression, we used an adenoviral vector carrying siRNA targeting human Nox4 (Adv-Nox4i) to silence the expression of Nox4 in rabbit conjunctival fibroblasts. As expected, Adv-Nox4i markedly reduced TGFβ1-stimulated Nox4 mRNA expression (Fig. 4A). Importantly, we also showed that Adv-Nox4i suppressed the production of H₂O₂ (Fig. 4B) and total collagen synthesis (Fig. 4C) in the presence of TGFβ1 stimulation. Similarly, removal of H₂O₂ using SOD/catalase mimetic EUK-134 also suppressed TGFβ1-induced total collagen production (Fig. 4D), suggesting that Nox4-derived H₂O₂ is required for collagen synthesis in conjunctival fibroblasts.

Curcumin Suppressed Generation of H₂O₂

In a cell-free aqueous solution (Krebs-Ringer buffer), direct exposure of H₂O₂ to Amplex Red and HRP increased red-fluorescent signal within 30 minutes. The H₂O₂-generated red-fluorescent signal was significantly decreased by curcumin in a concentration-dependent manner (Fig. 5A). In addition, we used an enzymatic reaction with xanthine (100 μM) and xanthine oxidase (0.03 U/mL) that caused a spontaneous dismutation of superoxide to H₂O₂ to assay the effect of curcumin. We also found that curcumin reduced xanthine/xanthine oxidase-induced H₂O₂ production in a concentration-dependent manner (Fig. 5B). These findings suggest that curcumin scavenges H₂O₂ from cell-free system.

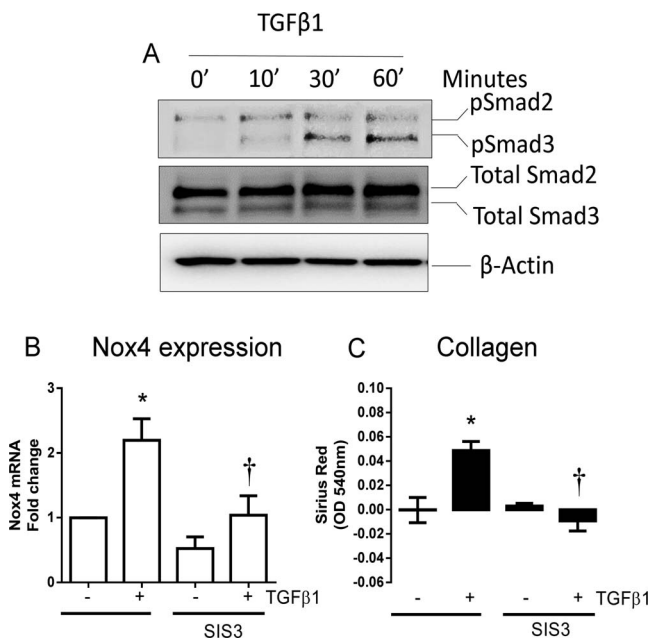


FIGURE 3. Effect of Smad3 inhibitor (SIS3) on Nox4 and collagen production in conjunctival fibroblasts. (A) TGFβ1 (5 ng/mL) induced Smad3 phosphorylation in the rabbit conjunctival fibroblasts. Pretreatment of Smad3 inhibitor SIS3 (5 μM) suppressed TGFβ1 (5 ng/mL) and induced (B) Nox4 gene expression (6 hours) and (C) total collagen expression (24 hours) in the rabbit conjunctival fibroblasts. All data are mean ± SEM from four to six independent experiments, **P* < 0.05 from Ctrl without treatment; †*P* < 0.05 from treated cells with TGFβ1.

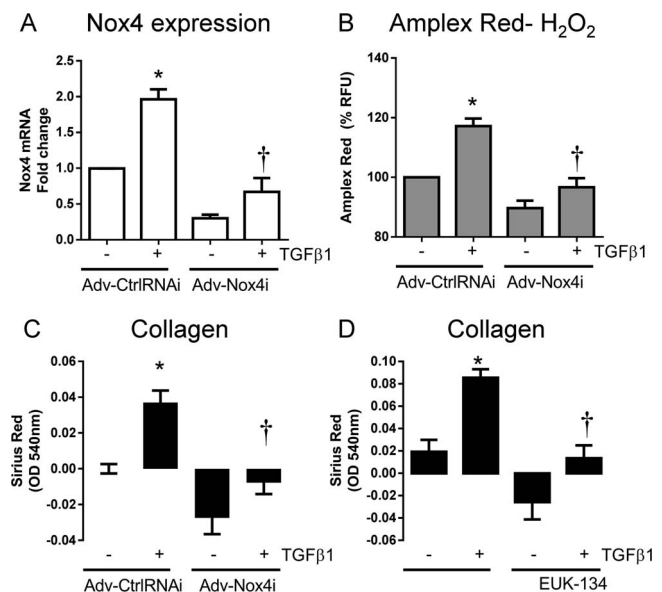


FIGURE 4. Effect of Nox4 inhibition on H₂O₂ and collagen production by conjunctival fibroblasts. Treatment of conjunctival fibroblasts with Adv-Nox4i inhibited the stimulatory effects of TGFβ1 (5 ng/mL) on Nox4 gene expression at 6 hours (A), generation of H₂O₂ at 6 hours (B), and total collagen at 24 hours (C). Similarly, H₂O₂ scavenger EUK-134 (25 μM) inhibited TGFβ1 (5 ng/mL)-induced total collagen production (D). All data are mean ± SEM from four to six independent experiments, **P* < 0.05 from control without treatment; †*P* < 0.05 from treated cells with TGFβ1.

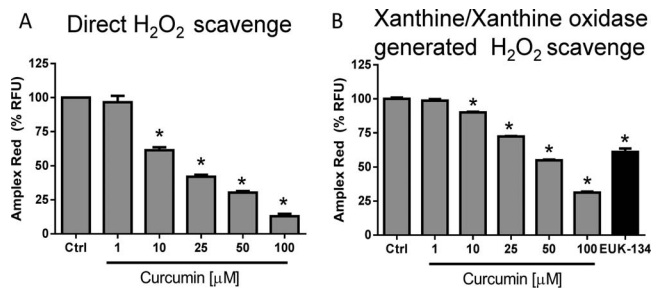


FIGURE 5. H₂O₂-scavenging effect of curcumin under cell-free system. Curcumin (10–100 μM) significantly suppressed Amplex Red fluorescent signal directly from H₂O₂ (A), generated enzymatically from xanthine (100 μM) and xanthine oxidase (0.03 U/mL; [B]) in a cell-free system. SOD/catalase mimetic EUK-134 (25 μM) inhibited Amplex Red fluorescent signal. All data are mean ± SEM from three to four independent experiments, **P* < 0.05 from Ctrl.

Curcumin Inhibits TGFβ1-Induced Nox4 Expression, H₂O₂ Generation, and Collagen Production

We finally investigated whether curcumin, apart from its antioxidant activity, can inhibit TGFβ1 induction of Nox4 expression and collagen production in conjunctival fibroblasts. To investigate the role of curcumin in TGFβ1-induced responses, conjunctival fibroblasts were treated with curcumin (10 μM) at 1 hour before stimulation with TGFβ1 (5 ng/mL). Interestingly, curcumin significantly suppressed TGFβ1-induced phosphorylation of Smad3 (Fig. 6A). Importantly, curcumin also abolished TGFβ1-induced Nox4 expression (Fig. 6B) and both production of H₂O₂ (Fig. 6C) and collagen (Fig. 6D). These findings suggest that curcumin blocks the activation of Smad3 and might have an inhibitory effect on expression of H₂O₂-producing enzyme Nox4 in addition to the antioxidant activity.

DISCUSSION

This study, for the first time, demonstrated that TGFβ1-induced Nox4 expression plays an important role in collagen synthesis by conjunctival fibroblasts. By inhibiting Smad3 with the pharmacologic inhibitor SIS3 or Nox4 expression with Adv-Nox4i, we showed that the stimulatory effect of TGFβ1 on collagen secretion by conjunctival fibroblasts is mediated via Smad3 and Nox4 pathway. In addition, this study also demonstrated that curcumin inhibits TGFβ1-induced Smad3 phosphorylation, Nox4 expression, and total collagen synthesis by conjunctival fibroblasts.

The NADPH oxidase family of ROS-generating enzymes is the key source of ROS in the vascular system and these enzyme complexes are crucial players in fibrosis.^{21,23,35} Five isoforms of the Nox catalytic subunit, namely, Nox1, Nox2, Nox3, Nox4, and Nox5, have been identified²⁵; and human pulmonary fibroblasts express both Nox1 and Nox4,³⁵ while human cardiac fibroblasts express Nox4 and Nox5.²¹ In contrast, the expression of Nox isoforms in fibroblasts derived from eyes and its implication in eye fibrosis have not been well studied. This is the first study to demonstrate that rabbit conjunctival fibroblasts express Nox2, Nox3, Nox4, and Nox5. Our findings are in line with the expression profile of Nox in human corneal stromal fibroblasts, which have mRNA encoding Nox2, Nox3, Nox4, and Nox5.³⁶ It appears that fibroblasts from various tissues such as heart,²¹ lungs,^{22,35} kidneys,²⁴ and cornea³⁶ express more than one subtype of Nox, but Nox4 tends to be the isoform involved in TGFβ1-mediated profibrotic responses at least in heart, lungs, and kidneys.^{21,24,35} The present study

also demonstrated that TGFβ1 did not stimulate the expression of Nox2, Nox3, and Nox5. Moreover, we showed that suppression of Nox4 gene expression with Adv-Nox4i abolishes TGFβ1 stimulation of total collagen synthesis, providing evidence that Nox4 is the subtype involved in TGFβ1-mediated fibrotic response in conjunctival fibroblasts. It remains to be investigated whether Nox4 is involved in profibrotic responses of TGFβ in animal models of ocular fibrosis; however, we have shown that inhibition of Nox4 with Adv-Nox4i attenuates TGFβ1-induced collagen accumulation *in vivo*.²⁰

Activation of Smad3 has been implicated in TGFβ1 stimulation of Nox4 and H₂O₂ formation in several cell types such as fibroblasts,^{22,37} human pulmonary artery smooth muscle cells,³⁸ and endothelial cells.³⁰ We also provided evidence that Nox4 expression requires TGFβ1-mediated activation of Smad3. We tested the effects of a specific inhibitor of Smad3 (SIS3), which has been shown to block the phosphorylation of Smad3 in rat kidney fibroblast cell line.²⁴ SIS3 completely attenuated TGFβ1-induced Nox4 expression and total collagen production in conjunctival fibroblasts, which is consistent to previous findings from rat kidney fibroblast cell line.²⁴ The present study highlights a role for Smad3 as an upstream messenger of TGFβ1 signaling pathway for the induction of Nox4 in conjunctival fibroblasts.

One of the mechanisms by which TGFβ1 promotes profibrotic responses by fibroblasts is via the generation of superoxide and H₂O₂.³⁹ TGFβ1 treatment increases the accumulation of ROS and the immunofluorescent expression of myofibroblast differentiation markers smooth muscle α-actin and F-actin by human corneal fibroblasts.⁴⁰ All of these TGFβ1-mediated responses are inhibited by the antioxidant N-acetylcysteine and diphenyleneiodonium, a nonselective Nox isoform pharmacologic inhibitor, thus highlighting involvement of NADPH oxidase-dependent ROS generation in human corneal fibroblasts.⁴⁰ Knocking down the gene expression of Nox4 has also been shown to reduce TGFβ1 induction of ROS generation, collagen release, and expression of smooth muscle α-actin by mouse cardiac fibroblasts²⁰ and rat kidney fibroblasts.²⁴ Likewise, the present study demonstrated that TGFβ1 elevates H₂O₂ production by conjunctival fibroblasts. The accumulation of TGFβ1-induced H₂O₂ is completely attenuated by Adv-Nox4i, thus suggesting a role of Nox4 in TGFβ1 induction of H₂O₂ generation by these cells. Unlike other Nox isoforms, Nox4 tends to primarily cause production of H₂O₂ rather than superoxide.⁴¹ Such a distinct functional property of Nox4 could be associated with the highly conserved extra cytosolic loop identified in Nox4 but not in other isoforms.⁴² We also demonstrated that TGFβ1 induction of Nox4-derived H₂O₂ plays an important role in collagen secretion by conjunctival fibroblasts. We therefore used Amplex Red, which is a known substrate for H₂O₂, to assay extracellular H₂O₂ generation by conjunctival fibroblasts.

The present study illustrated that curcumin suppresses H₂O₂ generation in a dose-dependent manner in a cell-free system. These findings are in line with the known antioxidant property of curcumin.⁴³ We also showed that curcumin inhibits phosphorylation of Smad3. Interestingly, in the absence of TGFβ1, curcumin also reduced collagen production without affecting basal level of H₂O₂ generation, suggesting that curcumin has an additional mechanism to regulate collagen production in the cells. In addition, curcumin inhibits p300HAT,²⁷ a key coactivator enzyme required for activation of several transcription factors such as Smad and NF-κB. We have recently shown that inhibition of p300 HAT activity with pharmacologic inhibitors such as garcinol and curcumin, or p300-specific siRNA, reduces both expression and activity of Nox4 in human endothelial cells.^{29,31} Consistent with our previous study, we demonstrated for the first time that curcumin reduces TGFβ1 induction of Nox4 gene expression

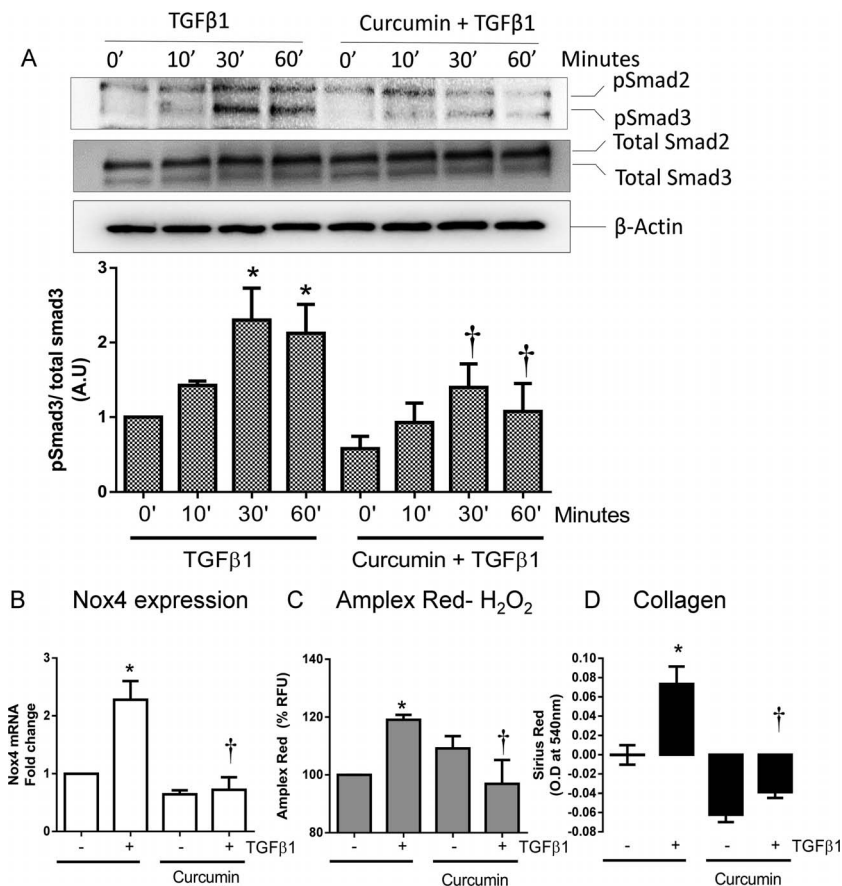


FIGURE 6. Effect of curcumin on Smad3 activation, Nox4 expression, production of H₂O₂, and collagen by conjunctival fibroblasts. **(A)** Representative Western blots showing inhibitory effect of curcumin (10 μM) on TGFβ1 (5 ng/mL)-induced protein expression of phosphorylated Smad3 and total Smad3 in conjunctival fibroblasts. **Bar chart** shows quantitative densitometry data measured and expressed in arbitrary unit (AU) by ImageJ software. Curcumin also inhibited the TGFβ1 (5 ng/mL)-induced Nox4 gene expression at 6 hours **(B)**, generation of H₂O₂ at 6 hours **(C)**, and total collagen at 24 hours **(D)**. All data are mean ± SEM from four to six independent experiments, **P* < 0.05 from control without treatment; †*P* < 0.05 from the cells treated with TGFβ1 (each time point in [A]).

by conjunctival fibroblasts, which would in turn attenuate H₂O₂ generation. Recently, TGFβ1 has been shown to increase Nox4 protein expression by human gingival fibroblasts, and such response is inhibited by a similar concentration of curcumin that is used in our study.⁴⁴ Collectively, the present study suggests that curcumin inhibits phosphorylation of Smad3, scavenges H₂O₂ generation, and suppresses expression of Nox4, which appears to be the main Nox isoform activated by TGFβ1; and these inhibitory effects of curcumin would decrease the subsequent release of collagen by these cells. The protective effect of curcumin appears to result from a combination of its antioxidant, anti-inflammatory, antiangiogenic, and antifibrotic properties and is attributable to the pleiotropic effects of curcumin on genes and effector molecules involved in these signaling pathways.^{26,28} Therefore, it would be of great interest to further explore the effect of curcumin in ocular fibrotic disorders in postsurgical complications and chemical burn injuries.²⁵

CONCLUSIONS

In conclusion, we demonstrated that TGFβ1-induced total collagen secretion by conjunctival fibroblasts is mediated via the Smad3/Nox4 pathway. Curcumin suppressed generation of H₂O₂ and Nox4 expression by these cells, which in turn dampened the secretion of total collagen after TGFβ1

treatment. Since conjunctival fibroblasts are one of the major contributors to ocular surface fibrosis associated with surgery or traumatic eye injury such as chemical burn, curcumin treatment might have therapeutic potential for these eye disorders.

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