NF-κB Mediates the Survival of Corneal Myofibroblast Induced by Angiotensin II

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Purpose. To investigate the role of angiotensin II (Ang II) in the regulation of corneal myofibroblast apoptosis and the possible signaling pathway.

Methods. Rabbit corneal myofibroblasts were cultured in vitro and the cell phenotype was identified by expression of α-smooth muscle actin (α-SMA) and formation of F-actin. The expression of Ang II type I receptor (AT1R) in keratocytes and corneal myofibroblasts were detected by immunofluorescence staining and Western blot. The effect of Ang II on corneal myofibroblast apoptosis induced by serum starvation and TNFα plus cycloheximide (CHX) was examined by TUNEL, Hoechst 33258 staining, and caspase 3/7 activity assay. The effect of Ang II on nuclear factor-κB (NF-κB)–dependent DNA binding activity and transcriptional activity was studied by electrophoresis mobility shift assay (EMSA) and luciferase reporter assay, respectively. Ang II–induced TGFβ1 secretion by corneal myofibroblasts was determined by ELISA.

Results. Ang II type I receptor expression was more abundant in corneal myofibroblasts compared with keratocytes. Ang II reduced corneal myofibroblasts apoptotic response to serum starvation or treatment with TNFα plus CHX. This protective effect was attenuated in the presence of AT1R antagonist losartan or NF-κB–specific inhibitor Bay11-7082. Ang II increased NF-κB–dependent DNA-binding activity and transcriptional activity, and also increased TGFβ1 production by corneal myofibroblasts.

Conclusions. Ang II induces corneal myofibroblasts resistance to apoptosis via activating NF-κB signaling pathway, and thus should be further investigated as a possible target for therapy of corneal fibrosis.

Keywords: angiotensin II, corneal myofibroblasts, NF-κB, apoptosis, corneal fibrosis

摘要

目的： 研究血管紧张素 II 对角膜肌成纤维细胞凋亡的调控作用及其信号通路

方法：体外培养兔角膜肌成纤维细胞并通过 α－SMA 和 F-actin 染色来确定细胞表型，利用免疫荧光技术和免疫印迹技术检测血管紧张素 II 的 I 型受体在角膜基质细胞和角膜肌成纤维细胞的表达，通过 TUNEL，Hoechst 33258 和 caspase3/7 活性检测来观察血管紧张素 II 对无血清培养和 TNFα/CHX 诱导的角膜肌成纤维细胞凋亡的作用，通过 EMSA 和双荧光素报告系统检测血管紧张素 II 对角膜肌成纤维细胞中 NF-κB 的 DNA 结合活性和转录活性的影响，通过 ELISA 检测血管紧张素 II 对角膜肌成纤维细胞分泌 TGFβ1 的作用。

结果：AT1R 在角膜肌成纤维的表达显著高于角膜基质细胞。血管紧张素 II 可以减少无血清培养和 TNFα/CHX 诱导的角膜肌成纤维细胞的凋亡，而这一作用可以被 AT1R 受体拮抗剂 losartan 和 NF-κB 抑制剂 Bay11-7082 所抑制。血管紧张素 II 可以提高角膜肌成纤维细胞中 NF-κB 的 DNA 结合活性和转录活性，并且可以促进角膜肌成纤维细胞分泌 TGFβ1。

结论：血管紧张素 II 可以通过激活 NF-κB 信号通路诱导角膜肌成纤维细胞抵抗凋亡，作为角膜纤维化的可能治疗靶点需要进一步的研究来证实。

关键词：血管紧张素 II，角膜肌成纤维细胞，NF-κB，凋亡，角膜纤维化

Cornea stroma is composed of highly ordered lamellae with sparsely distributed keratocytes. The keratocytes undergo phenotype transformation into fibroblasts and myofibroblasts in response to injury. Corneal myofibroblasts, characterized by intracellular expression of α-smooth muscle actin (α-SMA) and associated contractility that facilitates wound closure, play an important role in corneal fibrosis and scar formation. In normal condition, the myofibroblasts will diminish or disappear once the wound is closed. Persistent presence of corneal myofibroblasts is associated with corneal haze and pathologic fibrosis.[1–4] Apoptosis play an important role in the deletion of myofibroblasts during the wound-healing process, but the regulation of myofibroblast apoptosis is not well understood.[5,6]

Some cytokines such as IL-1 and TNFα are demonstrated to be involved in the regulation of corneal myofibroblast apoptosis, and stromal withdrawal of epithelial-derived TGFβ after restoration of basement membrane barrier function is also an important factor resulting in myofibroblast apoptosis.[7–10] However, the factors that favor the persistence presence of myofibroblasts in the stroma of cornea are not known well. Recent studies revealed that local renin-angiotensin system (RAS) play critical roles in tissue fibrosis such as liver, lung, and heart. Moreover, angiotensin II (Ang II) can be produced by activated and transformed hepatic myofibroblast and in turn favor the survival of myofibroblasts themselves in the liver fibrosis.[11,12] The presence of RAS and it role in corneal angiogenesis has been demonstrated by many studies, but so far there is no report regarding its possible effect in regulation of corneal myofibroblast apoptosis.

In the present study, we investigated the effects of Ang II on corneal myofibroblast apoptosis induced by serum starvation or cytokines in vitro. The possible role of nuclear factor–κB (NF-κB) signaling pathway in the action of Ang II was also studied.

Methods and Materials

Materials

Dulbecco's modified Eagle's medium (DMEM)/F12 medium, fetal bovine serum (FBS), 0.25% Trypsin-EDTA, were purchased from Invitrogen-Gibco (Carlsbad, CA, USA); 6-, 24-, and 96-well culture plates as well as cell culture flasks were from Corning (Corning, NY, USA). Recombinant human TNFα was purchased from PeproTech (Rocky Hill, NJ, USA). Ang II was from AnaSpec (San Jose, CA, USA), and losartan was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Collagenase, FITC-labeled phalloidin and 4′-6-diamidino-2-phenylindole (DAPI) were from Sigma Co. (St. Louis, MO, USA). Anti–α-SMA and anti-AT1R antibody were purchased from Abcam (Cambridge, UK). Anti-p65 antibody, horseradish peroxidase (HRP)-conjugated, and FITC-conjugated secondary antibody were purchased from Millipore (Billerica, MA, USA). Nuclear factor-κB inhibitor Bay11-7082, Hoechst 33258, cycloheximide (CHX) were from Bayotime Biotechnology Co., Ltd. (Beijing, China). DeadEnd Fluorometric TUNEL System, GL4.32[luc2P/NF-κB-RE/Hygro] Vector, pGL4.74[hRluc/TK] Vector, Dual-Luciferase Reporter Assay System, Fugene transfection reagent, and caspase-Glo 3/7 assay kit were purchased from Promega (Madison, WI, USA). LightShift Chemiluminescent electrophoresis mobility shift assay (EMSA) Kit, NE-PER Nuclear and Cytoplasmic Extraction Reagents, and Halt Protease Inhibitor Cocktail were from Pierce (Rockford, IL, USA). All reagent grade chemicals were from Sigma Co. unless otherwise indicated.

Cell Culture and Treatment. White New Zealand rabbits were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China). All the procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The epithelial and endothelial layers were removed from rabbit corneas using forceps and a scalpel blade. The stromal buttons were cut into small pieces and digested with 2 mg/mL collagenase in DMEM/F12 (1:1) overnight at 37°C. Isolated cells were collected by centrifugation, resuspended in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and cultured in the same medium, to transform the keratocytes to fibroblasts. Myofibroblasts were obtained from the subcultures of fibroblasts, which were passaged by trypsin-EDTA digestion. Myofibroblast phenotype was identified with immunofluorescence staining of α-SMA and F-actin. To obtain keratocytes, the isolated cell from collagenase digestion was seeded in serum free medium. To induce apoptosis, corneal myofibroblasts were subjected to serum starvation or treatment with TNFα plus CHX at the concentration as indicated.

Immunofluorescence. Cells were washed in PBS and fixed with 4% paraformaldehyde in phosphate buffer for 30 minutes at 4°C and permeabilized with 0.1% Triton X-100 solution for 5 minutes. All the following procedures were performed at room temperature. After three washes with PBS, the cells were incubated in 1% bovine serum albumin (BSA) in PBS for 30 minutes, to block nonspecific binding. Afterward, the cells were incubated for 1 hour with the corresponding primary antibodies at optimal dilutions in PBS containing 1% BSA. After they were washed PBS-BSA (three times, 5 minutes each), the cells were incubated with the corresponding secondary antibodies for 45 minutes. Fluorescein isothiocyanate–labeled phalloidin was used to stain F-actin, and DAPI was used to counterstain the nuclei. Negative controls were prepared using nonimmune serum instead of primary antibodies to exclude nonspecific staining.

Western Blot. Western blot was carried out as described previously.[13] Briefly, 50 μg of protein from cell lysates was mixed with sample buffer, separated on 10% SDS-PAGE, transferred to nitrocellulose membranes (Pall Corporation, East Hills, NY, USA) and then blocked overnight at 4°C in 5% defatted milk powder in Tris-buffered saline-0.1% Tween 20 (TBS-T). The membranes were probed with anti-AT1R primary antibodies, and subsequently washed with TBS-T and incubated with the secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) immunoblotting was performed as an internal control for equal loading. After extensive washing in TBS-T, the blots were developed with enhanced chemiluminescence reagents.

TUNEL Assay. Cells were grown on glass coverslips in 24-well plates, treated as described in the figure legend and fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. After this, the protocol was followed as recommended by the manufacturer (DeadEnd Fluorometric TUNEL System; Promega). 4′-6-diamidino-2-phenylindole counterstain was used to visualize nuclei. TUNEL-positive cells were scored in at least five fields for each coverslip, and at least 1000 cells were counted for each coverslip.

Hoechst 33258 Staining. Changes in the nuclear morphology of apoptotic cells were investigated by labeling the cells with the nuclear stain Hoechst 33258. Cells were seeded on glass coverslips in a 24-well plate for 24 hours. After treatment as indicated in the figure legend, the cells were washed twice with PBS and then fixed in 4% paraformaldehyde for 30 minutes. After two additional rinses with PBS, the cells were stained with 2 μM Hoechst 33258 in the dark for 10 minutes at room temperature. The cells were then washed twice with PBS and observed under a fluorescence micro-

scope. Apoptotic cells were identified by their characteristic nuclear condensation, fragmentation, and bright staining, whereas normal cells were identified by the normal, uniform chromatin pattern in their nuclei. The number of apoptotic cells is expressed as a percentage of total cells counted.

Caspase 3/7 Activity Assay. Cells were seeded in 96-well white-walled plates. After 24 hours, cells were treated with TNFα plus CHX to activate the caspases, with or without pretreatment of Ang II, AT1R antagonist, and NF-κB inhibitor. Caspase 3/7 activity was measured by Caspase-Glo 3/7 assay kit (Promega) according to the manufacturer's instructions. The luminescence of each sample was measured in triplicate with a microplate-reading luminometer (Safire II, Tecan, Germany).

Preparation of Nuclear Extracts. Nuclear fractions were collected by using NE-PER Nuclear and Cytoplasmic Extraction Reagents with a protease and phosphatase inhibitor cocktail (Pierce), as per the manufacturer's protocol. Nuclear protein concentrations were measured using Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

EMSA. Detection of DNA-protein binding by EMSA was done using LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce). The Biotin labeled NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGG ACTTTCCCAGGC-3′) was from Bayotime Biotechnology Co., Ltd. Binding reactions were performed by adding the nuclear extracts to a mixture containing biotin-labeled probes in binding buffer. The binding reactions were separated on a 6% PAGE in and subsequently electrophoretically transferred to a positively charged nylon membrane (Pall Biodyne B membrane; Pall Corporation, East Hills, NY, USA). After being blotted, the oligonucleotides were crosslinked for 10 minutes under UV-light. Avidin-HRP was applied after blocking and the biotinylated DNA was detected by enhanced chemiluminescence. A 200-fold excess of unlabeled consensus oligonucleotide (cold probe) was used for competitive binding to confirm the specificity of DNA–protein binding in the experiments. Cell cultures without treatment were used as control. Anti-p65 antibody was used for supershift assay.

Transient Transfection and Luciferase Activity. Corneal myofibroblasts were plated in each well of 96-well plates at a density of 5×10^3 cells in a final volume of 100 ul of culture medium, allowed to attach overnight and expand to 70% to 80% confluence. Transfection of NF-κB–driven firefly luciferase plasmid (pGL4.32[luc2P/NF-κB-RE/Hygro] Vector; Promega) and thymidine kinase-driven Renilla luciferase-expressing plasmid (pGL4.74[hRluc/TK] Vector; Promega) was carried out with Fugene HD transfection reagents (Promega) following the manufacturer's instructions. The transfected cells were cultured for 24 hours and then stimulated with Ang II for 6 hours with or without pretreatment of AT1R antagonist or NF-κB inhibitor. The cells were treated with passive lysis buffer (Promega), and cell extracts were collected for analysis of firefly and Renilla luciferase activities using the dual-luciferase reporter assay system (Promega). Both firefly and Renilla luciferase activity were measured using a Safire II Luminometer, and firefly luciferase activity was calculated relative to Renilla transfection efficiency. All measurements were performed in triplicate.

Enzyme-Linked Immunosorbent Assay. Corneal myofibroblasts were seeded into 24-well plates at the density of 1×10^4 cells/well and allowed to attach for 24 hours. The culture media was replaced with serum-deprived media to exclude the influence of the TGFβ1 from serum and then the cells were then treated with indicated concentrations of Ang II for the indicated time period. Untreated cells under similar culture conditions served as controls. Supernatant of cell cultures were collected and TGFβ1 concentrations released by corneal myofibroblasts were measured by a rabbit TGFβ1 ELISA kit (USCN Life Science Technology, Wuhan, China) according to the manufacturer's instructions. Cells released from the 24-well plate by trypsination were counted. Data are expressed as picogram/milliliter/1000 cells. Each sample was measured in triplicate.

Statistical Analysis. Results are expressed as means ± SD. Statistical analyses were performed using Student's *t*-test or, when multiple comparisons were made, one-way ANOVA with post hoc Tukey's test using SPSS (SPSS 11.5; SPPS, Chicago, IL, USA). Values of P less than 0.05 were considered statistically significant.

Results

Corneal Myofibroblast Culture and Phenotype Identification

The corneal keratocytes were isolated with collagenase digestion and then cultured in DMEM/F12 medium containing 10% FBS. After confluence, the cells were passaged with 0.25% trypsin-EDTA digestion for subculture. The cells at passage 3 were subjected to immunofluorescence staining of α-SMA and F-actin for identification of myofibroblast phenotype. As shown in Figures 1A and 1B, almost all the cells were positive for α-SMA and F-actin staining. The cells of passages 3 to 6 were used for subsequent experiments.

The Expression of AT1R in Corneal Keratocytes and Myofibroblasts

The expression of AT1R on corneal keratocytes and myofibroblasts were examined by indirect immunocytochemistry and Western blot. The keratocytes were isolated from corneal stroma and cultured in serum-free medium to maintain its phenotype. Corneal myofibroblasts demonstrated abundant AT1R expression, while less marked expression was observed in keratocytes (Fig. 2A). Considering the difference in cell size between these two kinds of cells, we also performed Western blot to quantitatively compare the expression of AT1R. The results of Western blot showed the expression of AT1R was higher in corneal myofibroblasts than in keratocytes (Fig. 2B).

Ang II Inhibited Corneal Myofibroblast Apoptosis Induced by Treatment of TNFα Plus CHX or Serum Starvation Via AT1R and NF-κB

Corneal myofibroblasts were subjected to serum starvation or treatment with TNFα plus CHX to induce cell apoptosis. The cells were pretreated with Ang II alone (100 nM) for 1 hour before apoptotic stimuli or combined with other reagents as indicated to examine the effect of Ang II on corneal myofibroblasts apoptosis. Cell cultures under same apoptotic stimuli without treatment of Ang II were used as control. To examine whether AT1R antagonist and NF-κB inhibitor have any effect on cell apoptosis, we also set groups of losartan alone and NF-κB alone. TUNEL assay and Hoechst 33258 staining were used to assess the apoptosis of the cells. For corneal myofibroblasts treated with TNFα plus CHX, Ang II treatment at the concentration of 100 nM reduced the apoptosis percentage from 25.5 ± 2.8% in control group to 14.2 ± 2.3% in Ang II–treated group, as determined with TUNEL assay (Fig. 3A), and from 27.7 ± 2.0% to 16.9 ± 1.9% as determined with Hoechst 33258 staining (Fig. 3C). For corneal myofibroblasts subjected to serum starvation, Ang II treatment at the concentration of 100 nM reduced the apoptosis percentage from 54.7 ± 6.6% in control group to 40.1 ± 3.9% in Ang II–treated group, as determined with TUNEL assay

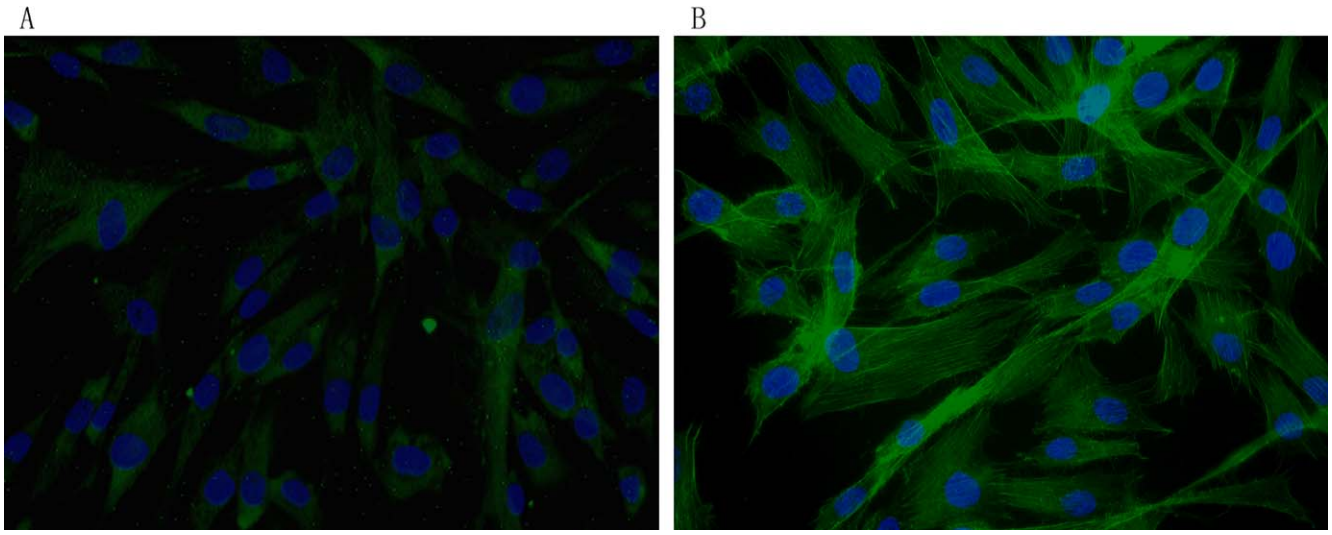

Figure 1. Expression of α-SMA and formation of F-actin in corneal myofibroblasts. The third passage corneal fibroblasts cultured on coverslips were immunostained with α-SMA or incubated with FITC-labeled phalloidin. The nuclei were counterstained with DAPI. (**A**) Colocalization of α-SMA (*green*) and nuclei (*blue*) in myofibroblasts. (**B**) Formation of F-actin in corneal myofibroblasts.

(Fig. 3B), and from 48.7 ± 4.1% to 35.3 ± 3.7% as determined with Hoechst 33258 staining (Fig. 3D). To evaluate the role of AT1R and NF-κB in Ang II–mediated protection from apoptosis, we used a specific AT1R antagonist losartan and NF-κB inhibitor Bay11-7082 in our experiments. Corneal myofibroblasts were pretreated with 10 μM losartan or 5 μM Bay11-7082 for 1 hour, respectively, and then exposed to Ang II and to the apoptotic stimuli. For cells subjected to serum starvation or treatment with TNFα plus CHX, the ratio of apoptotic cells were significantly increased in Ang II plus losartan group or Ang II plus Bay11-7082 group compared with Ang II–treated group as determined by TUNEL or Hoechst 33258 staining.

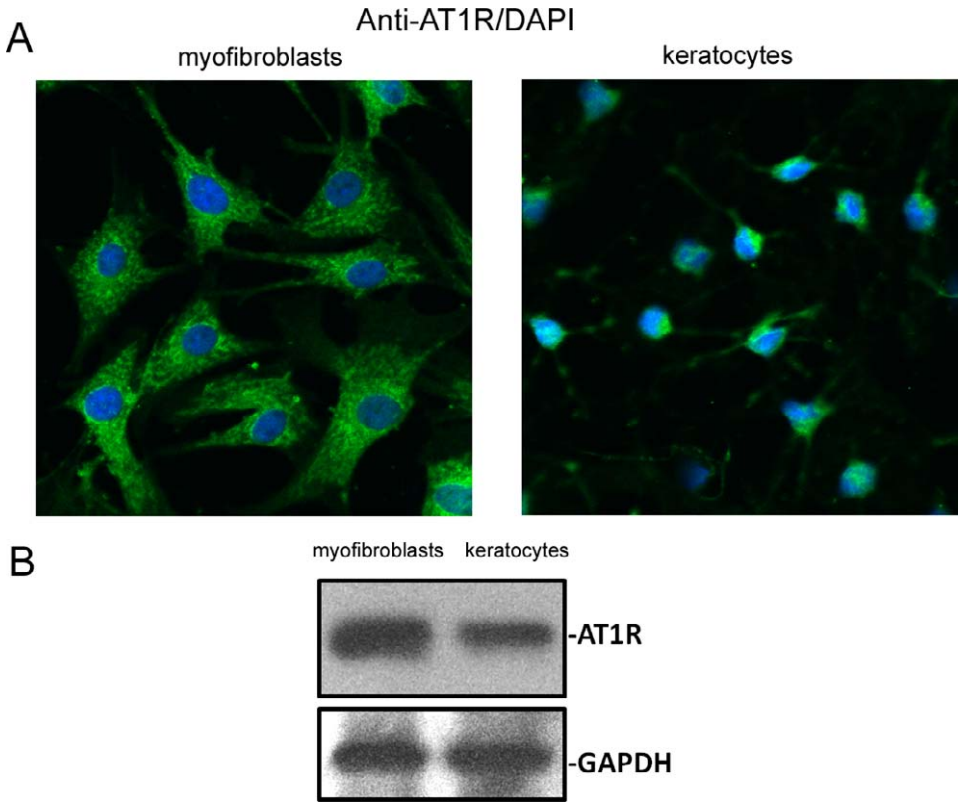

Figure 2. Expression of AT1R in corneal keratocytes and corneal myofibroblasts detected by immunofluorescence and Western blot. (**A**) Permeabilized corneal myofibroblasts and keratocytes were immunostained with AT1R antibodies (*green*). 4′-6-diamidino-2-phenylindole was used to counterstain the nuclei (*blue*). (**B**) Ang II type I receptor expression in corneal myofibroblasts and keratocytes was detected by Western blot. GAPDH immunoblots were used as control.

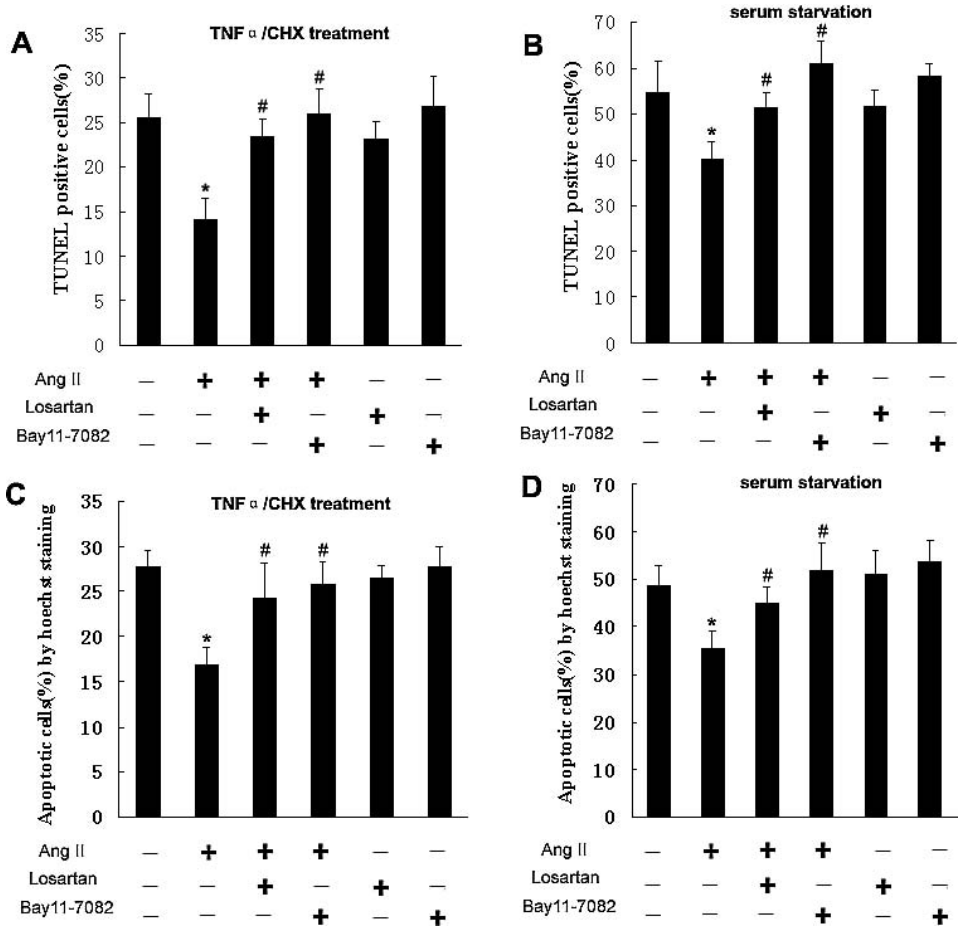

FIGURE 3. Effect of Ang II on corneal myofibroblasts apoptosis assayed by TUNEL and Hoechst 33258 staining. Corneal myofibroblasts were subjected starvation for 96 hours or treatment with TNFα (20 ng/mL) plus CHX (25 μg/mL) for 4 hours to induce apoptosis. Pretreatment of Ang II (100 nM) with or without AT1R antagonist, losartan (10 μM), or NF-κB inhibitor, Bay11-7082 (5 μM), as well as losartan or Bay11-7082 alone was applied to investigate the effect of ANG II on corneal myofibroblast apoptosis. Quantitative analysis of TUNEL for TNFα plus CHX treatment experiment (**A**) and serum starvation experiment (**B**) was performed by calculating the ratios between the number of TUNEL-positive cells and total cells counted. The percentage of apoptotic cells detected by Hoechst 33258 staining was calculated for TNFα plus CHX treatment experiment (**C**) and serum starvation experiment (**D**). The data are expressed as the means ± SD, $n = 5$. One-way ANOVA with post hoc Tukey test, $^*P < 0.05$ versus control; $^\#P < 0.05$ versus Ang II treatment.

Losartan alone or Bay11-7082 alone at the concentration indicated had no significant effect on the ratio of apoptotic cells compared with control groups as determined by TUNEL and Hoechst 33258 staining (Figs. 3A–D). These results demonstrated the involvement of AT1R and NF-κB in the effect of Ang II against apoptosis.

Ang II Inhibited Caspase 3/7 Activity in Corneal Myofibroblasts

To determine the effect of Ang II on the caspases activity in the process of corneal myofibroblast apoptosis, we investigated the caspase 3/7 activity by using a fluorimetric assay. Corneal myofibroblasts were treated with TNFα/CHX to activate caspases. Pretreatment with Ang II with or without combination of losartan or Bay11-7082 were used to investigate the role of Ang II on caspases activity. The results showed Ang II pretreatment at the concentrations of 100 nM and 1 μM significantly reduced the caspase3/7 activity to 65.7 ± 6.0% and 61.5 ± 7.4% of the TNFα/CHX-treated group (Fig. 4A). Addition of Losartan or Bay11-7082 on the basis of Ang II significantly increased the caspase 3/7 activity from 59.6 ± 5.3% to 87.1 ± 6.9% and 91.3 ± 6.3% of the TNFα/CHX-treated group, respectively. Losartan or Bay11-7082 alone had no significant effect on caspase 3/7 activity induced by TNFα/CHX (Fig. 4B).

Ang II Activated NF-κB in Corneal Myofibroblast

To investigate the possible role of NF-κB in the Ang II effect, we examined the effect of Ang II on NF-κB activation by measuring both NF-κB DNA-binding and NF-κB–dependent transcriptional activity. Our results showed that Ang II (100 nM) increased NF-κB–binding complex in corneal myofibroblasts after 15 minutes of treatment (Fig. 5A), and this effect was abrogated by pretreatment with AT1R antagonist losartan and NF-κB inhibitor Bay11-7082. Bay11-7082 alone obviously decreased NF-κB–binding complex compared with control, but losartan alone had no obvious effect on NF-κB binding activity (Fig. 5B).

We next investigated whether the Ang II–induced increase in NF-κB DNA binding corresponded with an increase in NF-κB–dependent gene transcription. Corneal myofibroblasts were transfected with a NF-κB–driven firefly luciferase plasmid and thymidine kinase–driven Renilla luciferase plasmid. Expo-

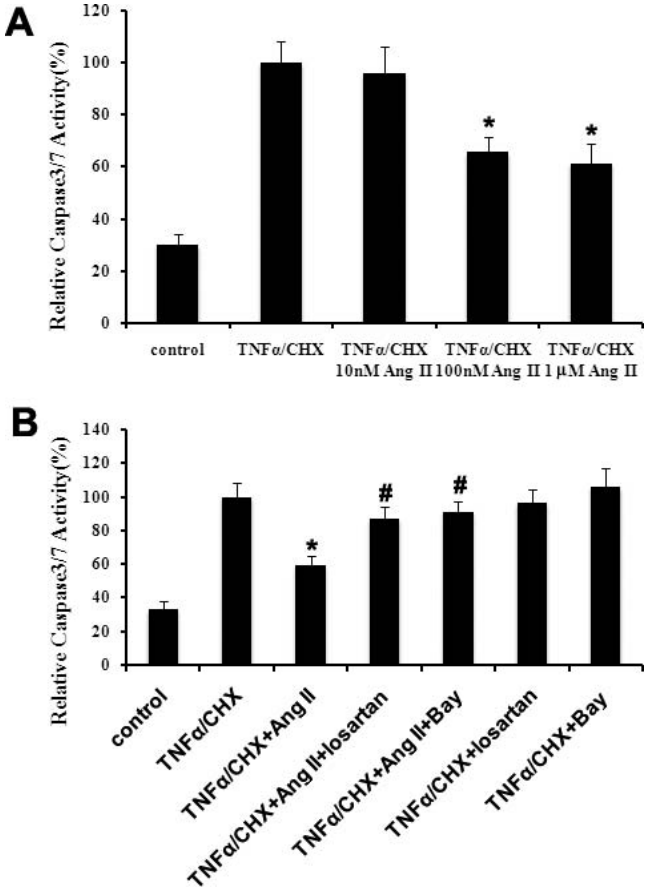

FIGURE 4. Ang II attenuated the increase of the caspase 3/7 activity induced by TNFα with CHX. Corneal myofibroblasts were cultured in 96-well plates and stimulated with TNFα (10 ng/mL) and CHX (10 μg/mL) for 6 hours. Caspase-3/7 activities were measured using the caspase-3/7 assay kit. (**A**) Effect of Ang II at different concentrations. (**B**) Effect of pretreatment with the AT1R antagonist losartan (10 μM) and NF-κB inhibitor Bay11-7082 (5 μM) on the action of Ang II (100 nM). Data are means ± SD, $n = 3$. One way ANOVA with post hoc Tukey test, $^{*}P < 0.05$ versus TNFα/CHX treated group; $^{\#}P < 0.05$ versus TNF/CHX plus Ang II–treated group.

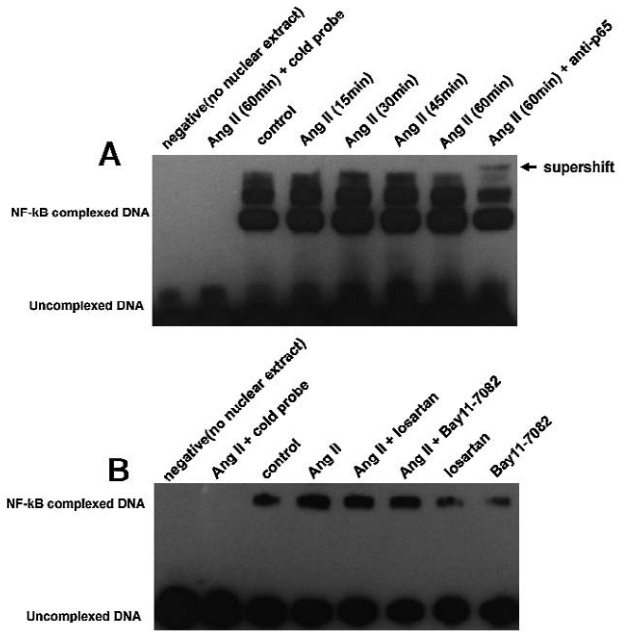

FIGURE 5. Effect of Ang II on NF-κB binding activity in corneal myofibroblasts. Nuclear extracts from corneal myofibroblasts were incubated with biotin end-labeled oligonucleotides containing the NF-κB consensus sequence and subjected to EMSA. (**A**) Time-course of NF-κB binding activity in corneal myofibroblast under Ang II (100 nM). (**B**) NF-κB binding activity in corneal myofibroblasts under Ang II (100 nM) for 1 hour with or without pretreatment of AT1R antagonist losartan (10 μM) or NF-κB inhibitor Bay11-7082 (5 μM), as well as under losartan (10 μM) or Bay11-7082 (5 μM) only. Corneal myofibroblasts without treatment were served as control. No nuclear extract was used as negative control. For competition experiments, a 200-fold excess of unlabeled probe (cold probe) was used. Anti-p65 antibody was used in supershift assay. Data are representative of at least three independent experiments.

sure of corneal myofibroblast to Ang II (100 nM) increased the NF-κB relative luciferase activity to 245.4 ± 28.7% of the control. Pretreatment with AT1R antagonist losartan or NF-κB inhibitor Bay11-7082 significantly attenuated the effect of Ang II, which decreased the NF-κB relative luciferase activity to 122.6 ± 11.5% and 97.1 ± 11.6% of the control, respectively. Losartan alone had no significant effect on NF-κB-relative luciferase activity. Bay11-7082 alone significantly decreased the NF-κB relative luciferase activity to 72.5 ± 6.6% of the control (Fig. 6).

Ang II Increased TGFβ1 Secretion in Corneal Myofibroblasts

Since TGFβ1 has been identified as a key mediator in corneal fibrogenesis, we determined the effect of Ang II on TGFβ1 production in corneal myofibroblasts with ELISA. We found that TGFβ1 concentration in culture medium was significantly increased by Ang II at the concentration of 100 nM and 1 μM to 31.4 ± 1.7 pg/mL/1000 cells and 33.8 ± 4.1 pg/mL/1000 cells, respectively, compared with 17.3 ± 1.7 pg/mL/1000 cells in control group (Fig. 7A). We further investigated the time course of Ang II effect, and the results showed TGFβ1 secretion began to increase at 12 hours after Ang II treatment and were markedly promoted at 24 hours (Fig. 7B).

DISCUSSION

The RAS consists of kidney renin that converts circulating plasma protein angiotensinogen into Ang I and angiotensin-converting enzyme (ACE) that converts Ang I to Ang II. Besides the circulating RAS, tissue-specific local RAS is also widely distributed in various tissues such as brain, heart, pancreas, kidney, blood vessels, lung, and liver. RAS has been well documented for its important role in regulation of blood pressure and plasma volume. Recent studies have demonstrated that RAS is involved in fibrosis in a variety of tissues, including heart, liver, lung, kidney, and skin.[12,14–19] The effect of Ang II in fibrosis is considered to be closely associated with TGFβ1. Ang II, produced locally by activated macrophages and fibroblasts in the wounded tissue, can directly stimulate TGFβ1 production and trigger fibroblast proliferation and differentiation into myofibroblasts. Moreover, it also stimulates its own production in myofibroblasts, thereby establishing an autocrine cycle of myofibroblast differentiation and activation. Therefore, the RAS system is believed to be potential targets for therapy of fibrosis in heart, liver, and kindey.[20]

However, little is known about the role of RAS in corneal fibrosis. Previous studies have showed the presence of RAS in

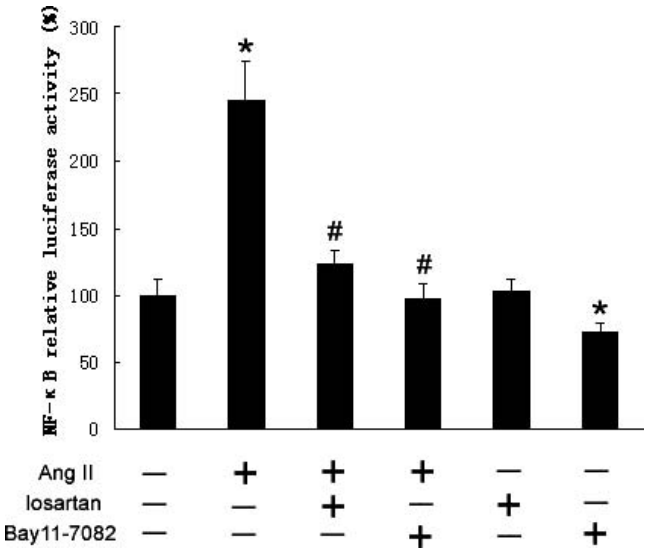

FIGURE 6. Ang II stimulation increased NF-κB-dependent transcriptional activity. Corneal myofibroblasts were cotransfected with a plasmid encoding firefly luciferase under the control of a NF-κB response element and with the transfection efficiency reporter pRL-TK Renilla luciferase. Transfected cells were treated with ANG II (100 nM) with or without pretreatment with AT1R antagonist losartan (10 μM) or NF-κB inhibitor Bay11-7082 (5 μM), as well as losartan or Bay11-7082 alone for 24 hours before measuring luciferase activity. The *graphs* show firefly luciferase normalized to Renilla luciferase as means ± SD of three repeats, and values are expressed in relative light units (RLU). One-way ANOVA with post hoc Tukey test, $^{*}P < 0.05$ versus control, $\#P < 0.05$ versus Ang II treated group.

cornea. ACE and Ang II were found to be present in human cornea, but the intensity of AT1R immunoreactivity was weak in normal cornea tissue.[21] The expression of Ang II and AT1R were markedly increased in the neovascularized mouse corneas compared with normal corneas.[22] The mRNA expression of ACE, AT1R, and AT2R was also found in corneal fibroblasts cultured in vitro.[23] These studies mainly focused on the possible role of local RAS in corneal angiogenesis and the results suggested ACE inhibitors might represent a novel therapeutic strategy to treat corneal neovascularization. To the best of our knowledge, this is the first study to investigate the role of Ang II in corneal fibrosis.

Many laboratory studies have demonstrated that corneal myofibroblasts develop from the progeny of keratocytes.[24,25] Bone marrow–derived cells might be another source of corneal myofibroblasts origin given the fact that studies had shown that bone marrow–derived cells served as precursors for myofibroblasts in other organs such as skin, liver, and lung, and this hypothesis was supported by experiments with chimeric mice model.[3,6] Apoptosis is thought to be the primary mechanism of myofibroblast disappearance in the process of cornea wound healing, despite the possibility that some myofibroblasts may undergo transdifferentiation back to corneal fibroblasts or keratocytes.[26] Two mechanisms are considered to be responsible for corneal myofibroblast apoptosis. Firstly, repair of corneal epithelial basement membrane and restoration of basement membrane barrier function lead to stromal withdrawal of epithelial-derived TGFβ, and possibly other cytokines such as platelet-derived growth factor (PDGF), which are important for phenotype transformation and viability maintenance of myofibroblast, and in turn induce corneal myofibroblast apoptosis.[10,27–30] Secondly, many cytokines including IL-1, TNFα, and platelet-activating factor (PAF) play active roles in the elimination of myofibroblasts by activating apoptosis related signaling pathway.[8,9] In this study, we used two different models of apoptosis, in which treatment with TNFα plus CHX or serum starvation was used, respectively, to imitate the in vivo condition that corneal myofibroblasts undergo apoptosis.

Our experiments showed Ang II reduced corneal myofibroblast apoptosis induced by serum starvation or treatment with TNFα plus CHX. The effect of Ang II was attenuated by AT1R specific antagonist losartan and NF-κB inhibitor Bay11-7082, and the results of EMSA and luciferase activity assay showed Ang II increased the DNA binding activity and transcriptional activity of NF-κB, indicating the involvement of AT1R and NF-κB signaling pathway in the action of Ang II. It has been reported that NF-κB modulates cell survival or apoptosis in many cell types through regulating expression of apoptosis associated proteins. Nuclear factor–κB acts as a homo- or

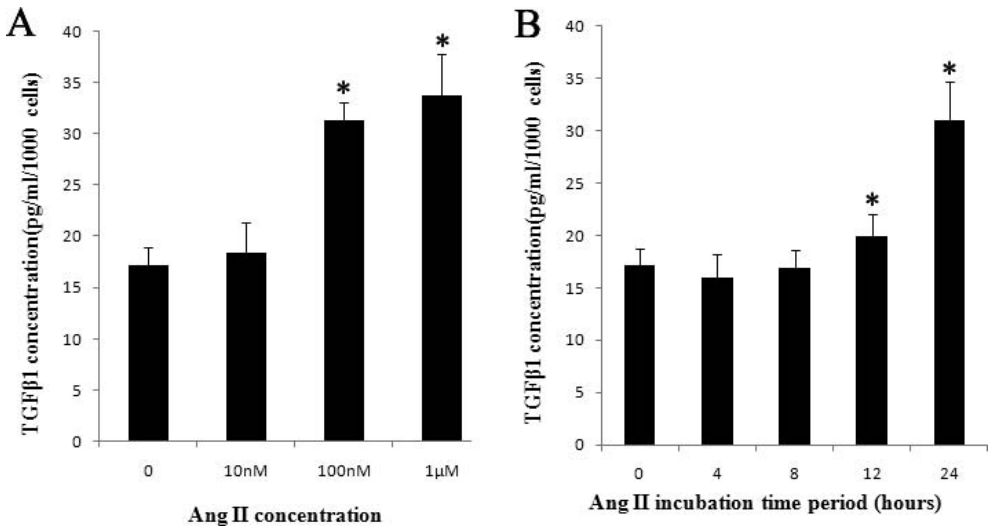

FIGURE 7. Ang II increased TGFβ1 secretion in corneal myofibroblasts. TGFβ1 concentration in culture supernatant was determined by ELISA. (**A**) Corneal myofibroblasts were treated with Ang II at different concentrations for 24 hours. (**B**) Corneal myofibroblasts were treated with Ang II at 100 nM for different time period. Data are means ± SD from three separate experiments performed under the same conditions ($^{*}P < 0.05$ versus untreated group by Student's *t*-test).

heterodimer composed of different combinations of the rel/NF-κB family member. The prominent form of NF-κB is p50/p65, which is located in the cytoplasm and bound to IκB family proteins. In condition of NF-κB activation, NF-κB dimmers are released from the NF-κB:IκB complex and translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes.[31] In such a way, NF-κB controls the transcription of genes favoring cell survival, including cellular inhibitors of apoptosis (cIAPs), BCL-2, TRAF1/TRAF2, and superoxide dismutase (SOD). Nuclear factor–κB was suggested to be a critical regulator of the survival of rodent and human hepatic myofibroblasts,[32] and NF-κB activation was also demonstrated to be important in regulation of TNFα-mediated corneal fibroblast apoptosis.[33] Consistent with previous studies, our study also suggests that NF-κB activation favors the survival of corneal myofibroblast.

The results of immunofluorescence and Western blot showed that the expression of AT1R is significantly more abundant in corneal myofibroblasts compared with that in keratocytes. We also found that Ang II increased TGFβ1 production, which has been suggested to be an important factor for the viability of corneal myofibroblast. Considering that the ACE activity has been demonstrated in corneal myofibroblast in vivo, we hypothesize that Ang II might exert it antiapoptotic effect via an autocrine way, which form a positive feedback loop and result in persistence presence of corneal myofibroblast. Since the secretion of TGFβ1 was markedly increased at 24 hours after Ang II stimulation, we think TGFβ1 might play an important role in the antiapoptotic effect of Ang II in the model of serum starvation, but not treatment with TNFα plus CHX.

Our results indicate that Ang II might play a role in corneal fibrosis, via inducing resistance to apoptosis in corneal myofibroblasts. Moreover, our experiments also support the involvement of NF-κB signaling pathway in the Ang II effect. These findings enrich our knowledge on the cellular and molecular mechanisms involved in corneal fibrosis, and suggest Ang II might be a potential target for the therapy of corneal haze or scar formation. However, further studies are required to investigate the regulation of local RAS system in cornea and the effect of RAS on corneal extracellular matrix in order to obtain an overall understanding of the role of RAS in corneal fibrosis.

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Investigative Ophthalmology & Visual Science

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