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
The Role of Ang II Regarding Corneal Myofibroblast

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 \( \alpha \)-smooth muscle actin (\( \alpha \)-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 TNF\( \alpha \) are demonstrated to be involved in the regulation of corneal myofibroblast apoptosis, and stromal withdrawal of epithelial-derived TGF\( \beta \) 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 role 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 its 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-\( \kappa \)B (NF-\( \kappa \)B) 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 TNF\( \alpha \) 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-\( \alpha \)-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-\( \kappa \)B inhibitor Bay11-7082, Hoechst 33258, cycloheximide (CHX) after restoration of basement membrane barrier function is also an important factor resulting in myofibroblast apoptosis.\(^{7-10}\) 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.\(^{5,6}\)

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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.

Corneal Myofibroblast Culture and Phenotype Identification

Corneal myofibroblasts 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-kB

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-kB inhibitor have any effect on cell apoptosis, we also set groups of losartan alone and NF-kB 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 42.22 ± 6.6% in control group to 40.1 ± 3.9% in Ang II–treated group, as determined with TUNEL assay.
(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 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.

**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.
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 was 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 caspase 3/7 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 Myofibroblasts**

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-
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 53.8 ± 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. 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 kidney.20

However, little is known about the role of RAS in corneal fibrosis. Previous studies have showed the presence of RAS in...
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. The expression of Ang II and AT1R were markedly increased in the neovascularized mouse corneas compared with normal corneas. The mRNA expression of ACE, AT1R, and AT2R was also found in corneal fibroblasts cultured in vitro. 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. 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. 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. 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. 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. 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. 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 6](http://iovs.journal])

**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.

![Figure 7](http://iovs.journal])

**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).
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heterodimer composed of different combinations of the rel/NF-kB family member. The prominent form of NF-kB is p50/p65, which is located in the cytoplasm and bound to IκB family proteins. In condition of NF-kB activation, NF-kB dimers are released from the NF-kB/IκB complex and translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes.33 In such a way, NF-kB 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.34 and NF-kB activation was also demonstrated to be important in regulation of TNFα-mediated corneal fibroblast apoptosis.35 Consistent with previous studies, our study also suggests that NF-kB 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 myofibroblasts. Considering that the ACE activity has been demonstrated in corneal myofibroblast in vivo, we hypothesize that Ang II might exert an antiapoptotic effect via an autocrine way, which form a positive feedback loop and result in permanent 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-kB 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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References


